REGULATION OF HTLV-I ONCOPROTEIN TAX BY PDLIM2

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Pengrong Yan

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This thesis/dissertation was presented

by

Pengrong Yan

It was defended on

Aug 5th, 2010

and approved by

Thomas Smithgall, Ph.D., Professor,
Department of Microbiology and Molecular Genetics

Patrick Moore, M.D. M.P.H., Professor,
Department of Microbiology and Molecular Genetics

Paul Robbins, Ph.D., Professor,
Department of Microbiology and Molecular Genetics

Jing Hu, Ph.D., Assistant Professor,
Department of Pharmacology and Chemical Biology

Thesis Director and Advisor: Gutian Xiao, Ph.D., Associate Professor,
Department of Microbiology and Molecular Genetics

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Pengrong Yan, PhD

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Pengrong Yan

University of Pittsburgh, 2010

Human T-cell leukemia virus type I (HTLV-I) is the etiological agent of adult T-cell leukemia (ATL). Its encoded oncoprotein Tax plays the key roles in HTLV-I-mediated cell transformation and pathogenesis. Although the mechanisms by which the HTLV-I Tax deregulates cellular signaling for oncogenesis have been extensively studied, how Tax itself is regulated remains largely unknown.

Here we showed that PDZ-LIM domain-containing protein 2 (PDLIM2, SLIM or Mystique) negatively regulated Tax by promoting poly-ubiquitination and proteasomal degradation of Tax, so that to suppress Tax-mediated signaling activation, cell transformation and oncogenesis both *in vitro* and in animal. To further define the molecular determinant responsible for PDLIM2 mediated Tax suppression, we characterized that a putative α-helix motif of PDLIM2 at amino acids 236-254 was crucial for the interaction between PDLIM2 and Tax. PDLIM2 with selective disruption of this short helix lost the tumor suppression function and failed in altering Tax subcellular distribution as well as promoting Tax proteasomal degradation. Additionally, the expression of PDLIM2 was down-regulated in HTLV-I-transformed T cells and primary ATL samples, and the re-introduction of PDLIM2 reversed the

tumorigenicity of the malignant cells. The evidence indicated that the counterbalance between HTLV-I Tax and PDLIM2 might determine the outcome of HTLV-I infection. Meanwhile, in those HTLV-I-transformed T cells, we found that DNA methyltransferases (DNMT) 1 and 3b but not 3a were over-expressed, suggesting the involvement of DNA methylation in PDLIM2 repression.

Consistently, the DNMT inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) restored PDLIM2 expression and induced death of these malignant cells. Our studies provided important insights into the function of PDLIM2 in HTLV-I leukemogenicity, long latency and cancer heath disparities. Given the efficient antitumor activity with no obvious toxicity of 5-aza-dC, our studies also suggest potential therapeutic approaches for ATL, a disease with poor treatments.

This thesis is dedicated to my parents, my parents-in-law and

my wife, Jin,

for their unconditional support through all my life.

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1.0 GENERAL INTRODUCTION

Adult T cell leukemia (ATL) was initially described in the mid 1970s (Takatsuki, 2005; Yoshida, 2005, 2010). In 1980 and 1981, investigators from Japan and USA independently isolated human T cell leukemia virus type I (HTLV-I), the first human retrovirus to be described (Gallo, 2005; Grassmann *et al.*, 2005; Kashanchi and Brady, 2005; Yoshida, 2005). It was found to be the causative agent of ATL, a clonal aggressive and fatal malignancy of CD4+ T cells (Yoshida *et al.*, 1984). Additionally, HTLV-I infection results in a distinct neurological disorder termed HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al.*, 1985; Osame *et al.*, 1986). Overcoming the immune responses as well as deploying intracellular signaling, is critical for HTLV-I-mediated cell transformation and ATL development.

Unlike many other transforming retroviruses, HTLV-I does not have viral homologues of cellular proto-oncogenes. Instead, the nonstructural oncoprotein Tax, encoded by the HTLV-I pX region (Figure 1), contributes to the primary transforming and long-term persistent infection (Seiki *et al.*, 1983; Seiki *et al.*, 1982). Tax protein is unique for HTLV-I and exerts its oncogenic role largely through deregulation of cellular transcription factors that are critical for cell growth and division, such as NF-κB (Sun and Yamaoka, 2005).

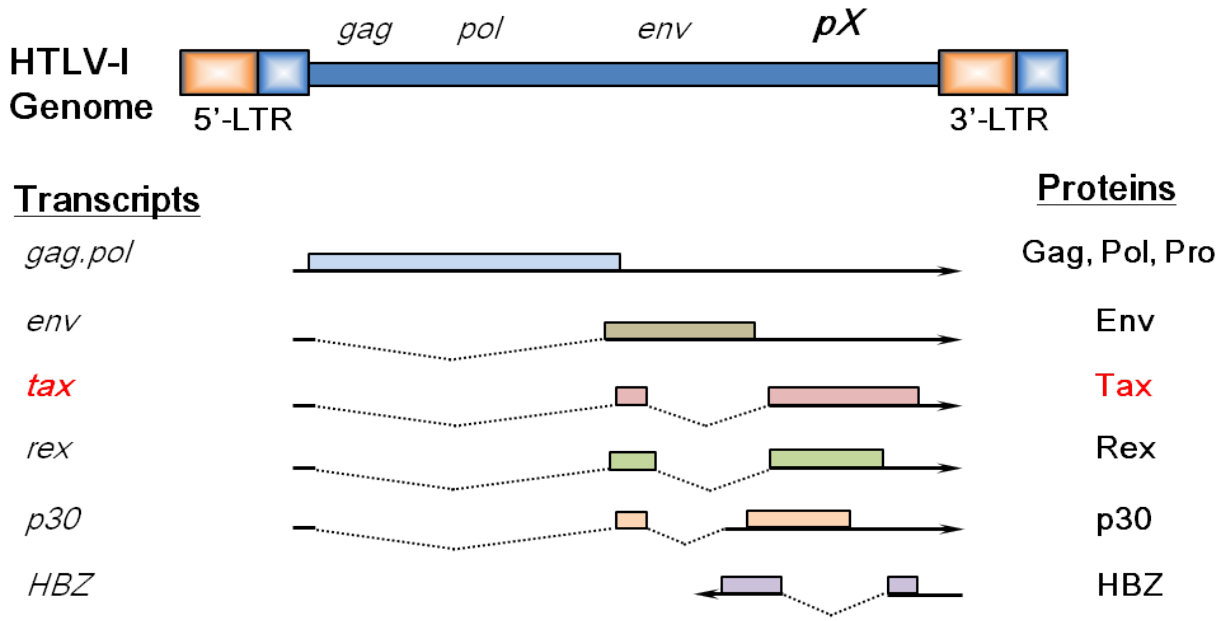

Figure 1. Scheme of the HTLV-I genome and HTLV-I transcription map.

The alternatively spliced mRNAs, and proteins encoded by each mRNA are shown. ORFs are indicated by the boxes.

The mechanisms by which HTLV-I infects host T cells and induces cell transformation have been extensively studied in the past thirty years (Hall and Fujii, 2005; Satou and Matsuoka, 2010; Yoshida, 2010). HTLV-I-induced ATL is an NF-κB related disease. Evidence showed that: 1) ATL cells exhibit constitutive activation of NF-κB and resistance to apoptosis (Horie, 2007; Mori *et al.*, 1999). 2) Transduction of super-suppressor form of Inhibitor of NF-κB (IκB) results in the promoted cell apoptosis and reversed malignant phenotype of ATL cells (Hironaka *et al.*, 2004). 3) Tax is required for HTLV-I-mediated cell transformation, and Tax-mediated NF-κB activation is essential for the tumorigenesis in some skin diseases (Kwon *et al.*, 2005). 4) Additionally, chemical inhibition of NF-κB induces apoptosis of HTLV-I-infected cells, supporting the pivotal role of NF-κB signaling in maintaining HTLV-I infection and ATL development (Horie, 2007; Uozumi, 2010). In the following sections, I will highlight

observations on the infectivity of HTLV-I as well as the implications of oncoprotein Tax in HTLV-I-mediated pathogenesis. Also, based on the involvement of NF-κB, I will summarize functions of the NF-κB signaling in tumorigenesis and discuss the regulation mechanisms of NF-κB, especially via PDZ-LIM domain-containing protein 2 (PDLIM2, SLIM or Mystique), a newly defined negative regulator of NF-κB.

1.1 HTLV-I INFECTIVITY AND IMPLICATIONS OF HTLV-I TAX IN HTLV-I-MEDIATED PATHOGENESIS

1.1.1 Clinical features of ATL and infectivity of HTLV-I

1.1.1.1 HTLV-I prevalence and the clinical features of ATL

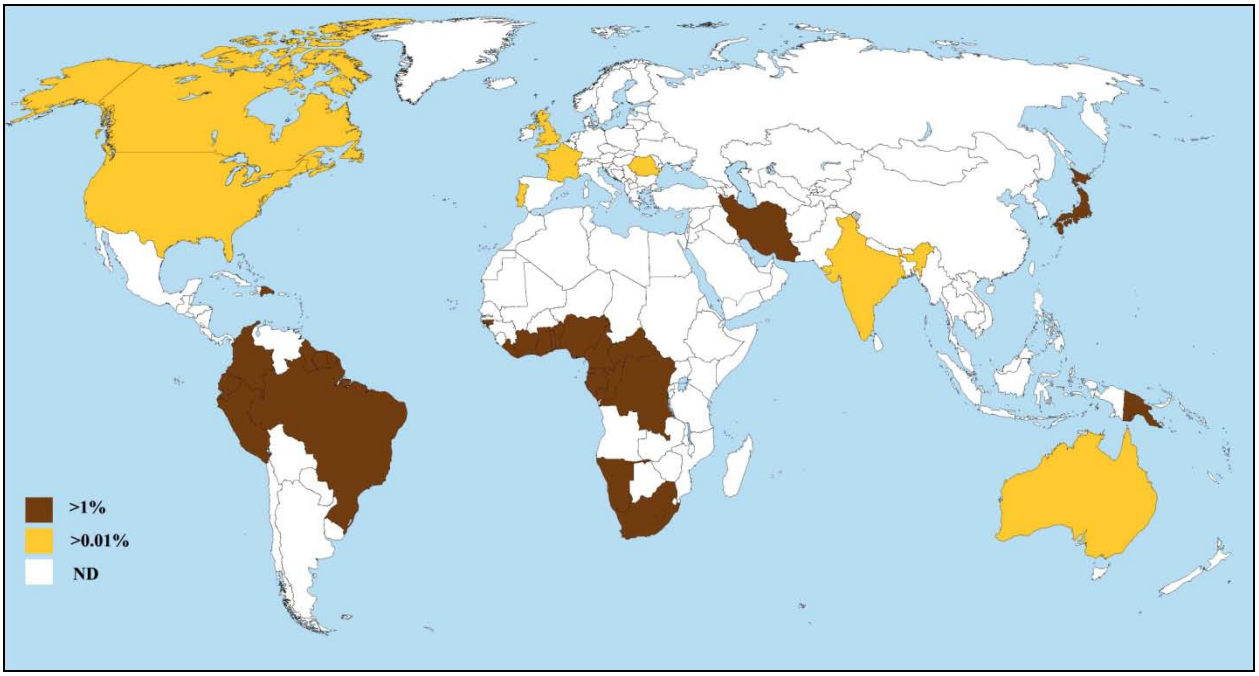

Figure 2. The prevalence of HTLV-I infection.

Countries in dark brown have HTLV-I prevalence between 1 and 5% in some populations. Countries with reports of low prevalence (less than 1% in some groups), due mainly to immigration from endemic areas, are shown in tan color. Remaining countries with very low HTLV-I prevalence or unreported data are presented in white (Proietti *et al.*, 2005).

HTLV-I was the first human retrovirus to be discovered. Today, 15-20 million people are infected worldwide. Its prevalence is endemic in certain areas (Figure 2), especially in

southwestern Japan, parts of Africa and South America, where 2~10% of regional population is infected (Proietti *et al.*, 2005). In Australia, the prevalence of HTLV-I is estimated to be up to 14% in the central region, compared to 4.7% in the Northern Territory cattle country - and 0.5% in Darwin (Northern Territory Government, 2005). Because HTLV-I infection is chronic if left untreated, the number of infected people keeps increasing as HTLV-I infection becomes a worldwide health threat.

Evidence suggests that HTLV-I is the causative agent. Indeed: 1) ATL develops only in HTLV-I-infected individuals, and all ATL cells contain integrated HTLV-I provirus (Yoshida, 1984). 2) HTLV-I randomly integrates into the host genome of ATL patients. 3) During the latency period of ATL development, the HTLV-I-infected cells transitions from polyclonal to monoclonal expansion, supporting the etiological role of HTLV-I. Consistently, the integration loci of the HTLV-I provirus are specific and clonal for each patient (Yoshida *et al.*, 1984). 4) The HTLV-I-specific pX region (Figure 1), which encodes Tax (the key factor in the tumoriginecity of HTLV-I), does not share homologous sequences with host cellular DNA (Yoshida, 2010).

The cumulative risks of developing ATL among virus carriers are estimated to be 2-5 % following a 40-60-year post-infection latency period, whereas the majority of HTLV-I carriers remain asymptomatic (Yoshida, 2010). Significantly, HTLV-I infection is associated with the formation of several autoimmune diseases, increases the risk of primary malignant neoplasms, and promotes the development of diseases including acquired immune deficiency syndrome (AIDS), when it co-infects individuals with human immunodeficiency virus (HIV) (Goorney and Young, 2000; Gotuzzo *et al.*, 1992; Nadler *et al.*, 1996). Thus, although not all HTLV-I

infections are life-threatening, the incident effects should be emphasized when considering the prevention and diagnosis of many other diseases.

ATL is composed of four subtypes: chronic, smoldering, acute and lymphomatous. The latter two present fatal and aggressive clinical phenotypes, while the former two appear to be indolent and cause little or no morbidity. Reports suggest median survival periods of 6.2 month for the acute type, and 10.2 months for the lymphoma type following diagnosis (Shimoyama, 1991). “Flower cells”- or the multi-nucleated leukemic cells associated with aneuploidy and chromosomal instability, are the main clinical markers of ATL (Uchiyama *et al.*, 1977). ATL development is associated with the infiltration of flower cells into various tissues including skin. Further analysis has revealed aberrantly elevated levels of interleukin 2-receptor (IL-2R, CD25) and p52, the mature form of NFκB2 in those ATL cells, indicating the involvement of impaired cellular signaling during ATL progression (Hattori *et al.*, 1981).

1.1.1.2 Unique infectivity and transmission of HTLV-I

HTLV-I primarily infects- but is not limited to- immune cells, including T cells, B cells, endothelial cells, glial cells, mammary epithelial cells and monocytes of both human and non-human origin, but it preferentially targets the memory subpopulation of CD4+ T lymphocytes *in vivo* (Akagi *et al.*, 1992; Ho *et al.*, 1984; Hoffman *et al.*, 1992; Hoxie *et al.*, 1984; Koyanagi *et al.*, 1993; LeVasseur *et al.*, 1998; Richardson *et al.*, 1990). This infection pattern is consistent with the ubiquitous expression of HTLV-I receptors, the glucose transporter 1 protein (GLUT1) and heparin proteoglycan, in those cell types (Manel *et al.*, 2005; Manel *et al.*, 2003). There are three principle methods of HTLV-I transmission: mother to infant (transmission of infected T

cells and macrophages via breast milk feeding), sexual contact and parenteral transmission. In all cases, HTLV-I is transmitted through cell-cell contacts via virological synapses. In other words, infected cells must be passed from infected individuals, in contrast to cell-free viruses, which are largely non-infectious (Igakura *et al.*, 2003; Matsuoka and Jeang, 2007). More specifically, the HTLV-I Tax protein contributes to cell–cell contact by promoting the formation of virological synapses thereby favoring the infection process (Barnard *et al.*, 2005; Igakura *et al.*, 2003; Matsuoka and Jeang, 2007; Nejmeddine *et al.*, 2005)**.**

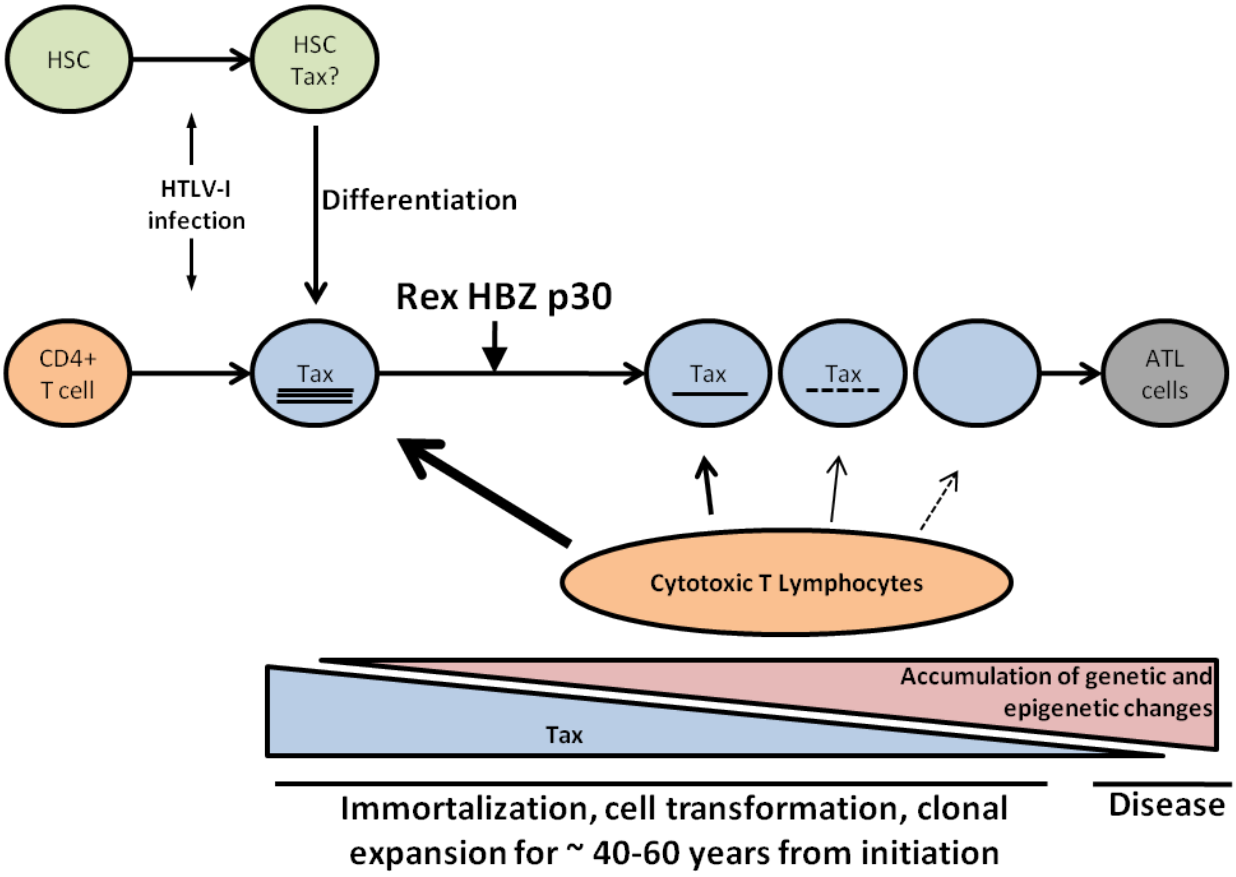

Figure 3. ATL progression from HTLV-I infection.

It is important to note that- the population of HTLV-I-infected cells depends on a persistent clonal proliferation *in vivo* (Figure 3), indicating a causative role of HTLV-I in ATL

progression (Etoh *et al.*, 1997; Etoh *et al.*, 1999; Yoshida, 2010). Following infection, the HTLV-I provirus load increases through the proliferation of infected cells rather than by the lysis of host cells and results in the release of mature viruses. The cytotoxic T lymphocyte (CTL) response also contributes to the clonal expansion because CTLs recognize and kill most of the HTLV-I-infected cells, leaving only a small portion of infected cells, that escape and/or overcome CTL responses and can later expand (Bangham and Osame, 2005; Yoshida, 2010). Importantly, although the *de novo* infection of HTLV-I is observed and is crucial in initiating HTLV-I-mediated leukemogenesis, some evidence has challenged the role of *de novo* spreading in the HTLV-I provirus load after the initial infection. Studies on inhibitors of reverse transcriptase (RTIs) indicate that after RTI treatments, the provirus load merely changes in both immediate HTLV-I-infected cells and the cells from HAM/TSP patients, in correlation with the clonal expansion of infected cells in HTLV-I-mediated pathogenesis (Miyazato *et al.*, 2006; Taylor *et al.*, 2006).

1.1.1.3 Unique viral replication of HTLV-I

There are roughly three periods of HTLV-I replication, depending on the splicing states of HTLV-I mRNA. The proteins Tax and Rex, which are both encoded by the pX region (Figure 1), predominantly cooperate to contribute to HTLV-I viral replication (Fujisawa *et al.*, 1985; Hidaka *et al.*, 1988; Inoue *et al.*, 1987; Seiki *et al.*, 1985; Sodroski *et al.*, 1984). During the first stage, HTLV-I pre-mRNA is automatically fully spliced, resulting in the expression of both Tax and Rex. Tax is a transactivator protein that promotes HTLV-I viral transcription, resulting in an elevated production of HTLV-I pre-mRNAs through binding of the Tax responsive element (TRE) within the HTLV-I 5' long terminal repeat (LTR). Rex functions to inhibit the splicing of

pre-mRNAs and maintain the unspliced and singly spliced forms, which encode the HTLV-I particle proteins including Gag, Pol and Env (Figure 4). In other words, the expression of Rex promotes the production of mature mRNA encoding HTLV-I particle proteins, while simultaneously inhibiting the generation of Tax and Rex mRNAs.

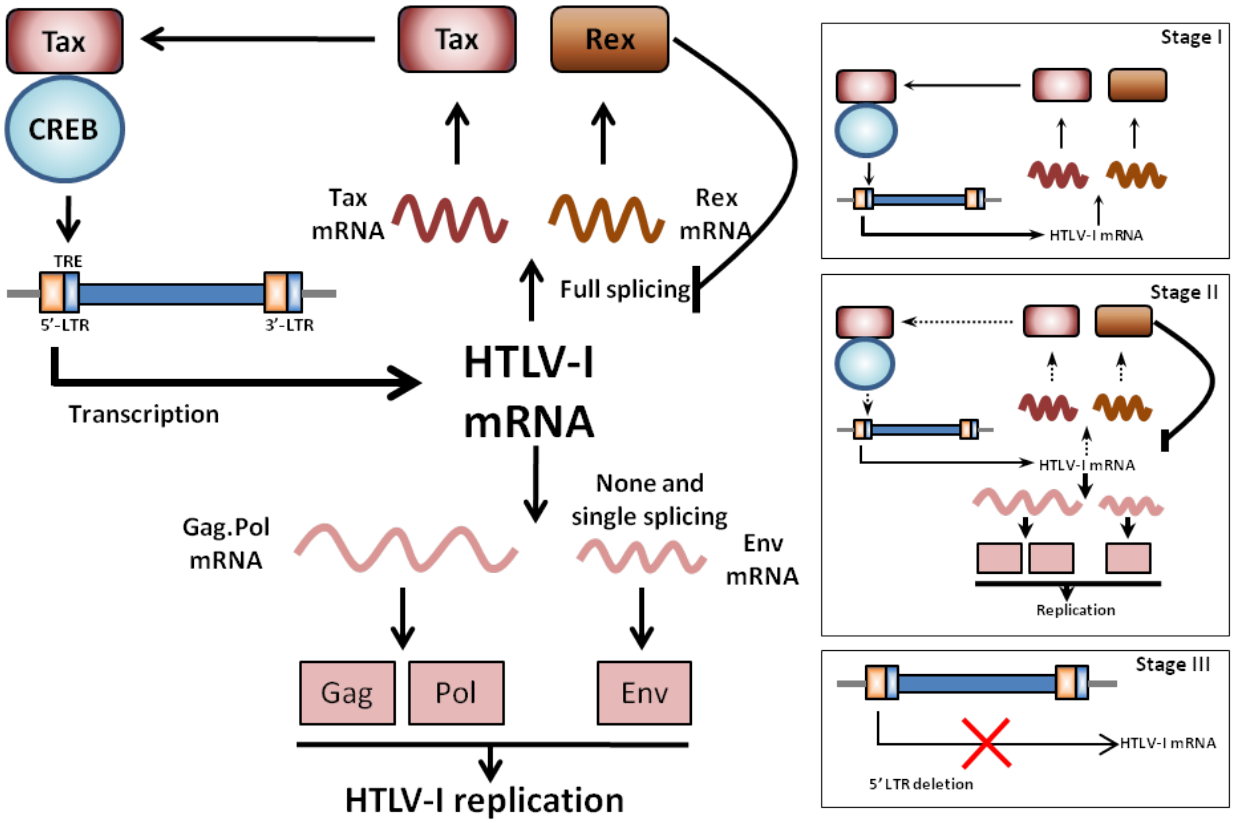

Figure 4. Regulation of HTLV-I replication by Tax and Rex.

In the second phase, the unspliced and singly spliced HTLV-I mRNAs are maintained in abundance by the accumulation of Rex, resulting in efficient translations and the assembly of particle proteins to promote viral replication. Meanwhile, due to the suppressed production of Tax mRNA, insufficient Tax protein is translated. Gradually, the repressed Tax expression leads to the shutdown of HTLV-I viral transcription and the inhibition of particle protein expression,

and resulting in the suppression of viral replication. Therefore, HTLV-I viral transcription and replication are transient processes.

In accordance with the clonal expansion of HTLV-I-infected cells in ATL, a correlation between the silencing of Tax and the shutdown of HTLV-I replication is frequently observed, thus defining the third period. The HTLV-I 5' LTR is frequently deleted or hyper-methylated during ATL progression, highlighting the importance of suppressed viral transcription in the late stage of HTLV-I infection (Satou *et al.*, 2006). This state is accepted to contribute to the latency between HTLV-I infection and ATL development because it helps HTLV-I-infected cells to escape CTL responses predominantly targeting Tax. Following a long latency period and clonal expansion of the HTLV-I-infected cells, ATL manifests itself through the accumulation of infected cells, breaking the threshold of immune responses (Figure 3).

In summary, HTLV-I is a retrovirus responsible for ATL generation that has unique clinical characteristics and infection and replication processes. Importantly, the viral-encoded oncoprotein Tax contributes to both the viral infection and replication by favoring the formation of virological synapses and promoting HTLV-I transcription, respectively.

1.1.2 Host responses to HTLV-I infection

The host exhibits two main responses to HTLV-I infection: the cellular immune response to HTLV-I-infected cells, and the intracellular signaling responses, such as the NF-κB pathway (Bangham and Osame, 2005; Hall and Fujii, 2005; Matsuoka and Jeang, 2007; Yoshida, 2010).

The immune response to HTLV-I, especially the CTL response, is involved in determining the proviral load of HTLV-I and the risk of disease progression, predominantly

through the targeting of HTLV-I Tax (Goon and Bangham, 2004; Jacobson *et al.*, 1990; Parker *et al.*, 1992; Satou and Matsuoka, 2010). Importantly, the contributions of the immune response to ATL development are strongly supported by a recent ATL model using HTLV-I-infected hematopoietic stem cells (HSCs) in SCID mice (Banerjee *et al.*, 2010b). These mice exhibit phenotypes similar to ATL, with the characteristic genetic, immunologic, histologic, pathologic and clinical features of ATL. Furthermore, under these immune-deficient conditions, the mice exclusively generate lymphomas with a low number of injected cells (as few as 100 cells), at high rates (43%) and with short latency (~17 weeks), demonstrating the crucial role of immune repression in ATL development.

Post HTLV-I infection, HTLV-I specific CTLs are highly activated *in vivo* and the host presents strong anti-HTLV-I responses (Vine *et al.*, 2004). However, the CTL response is genetically determined and depends on individual differences (Jeffery *et al.*, 1999; Nagai *et al.*, 1998; Vine *et al.*, 2002). These differences in response partially explain the different clinical manifestations in HTLV-I-infected individuals with similar provirus loads. Natural killer (NK) cells and CD4+ helper T cells are also involved in the response to HTLV-I infection (Goon and Bangham, 2004). It has been reported that HAM/TSP patients exhibit both lower frequencies and lower activities of NK cells than asymptomatic HTLV-I carriers (Fujihara *et al.*, 1991; Yu *et al.*, 1991). Additionally, the portion of interferon (IFN) γ producing CD4+ T cells is 10-25 times higher in HAM/TSP patients than in the carriers, suggesting a contribution from CD4+ cells through the secretion of anti-viral cytokines (Goon *et al.*, 2003; Goon *et al.*, 2004). Like infections from other viruses, HTLV-I infection is associated with HTLV-I antibody production and elevated activated T cells levels, which result in inflammatory tissue damage. Such stresses

provide a wide negative selection for the HTLV-I-infected cells that are insensitive to those anti-HTLV-I responses, facilitating ATL progression though the clonal expansion of the escaped cells.

On the other hand, at the intracellular level, HTLV-I infection impairs signaling cascades including NF-κB, AP-1, JAK/STAT and TGF-β (Hall and Fujii, 2005; Matsuoka and Jeang, 2007; Sun and Yamaoka, 2005; Yoshida, 2010). Such deregulation provides survival signals and inactivates multiple tumor suppressor genes, allowing cells to escape from apoptosis and promote cellular proliferation and transformation. Importantly, the Tax protein plays a central role in all those processes, as summarized below.

1.1.3 Role of the Tax oncoprotein in HTLV-I pathogenesis

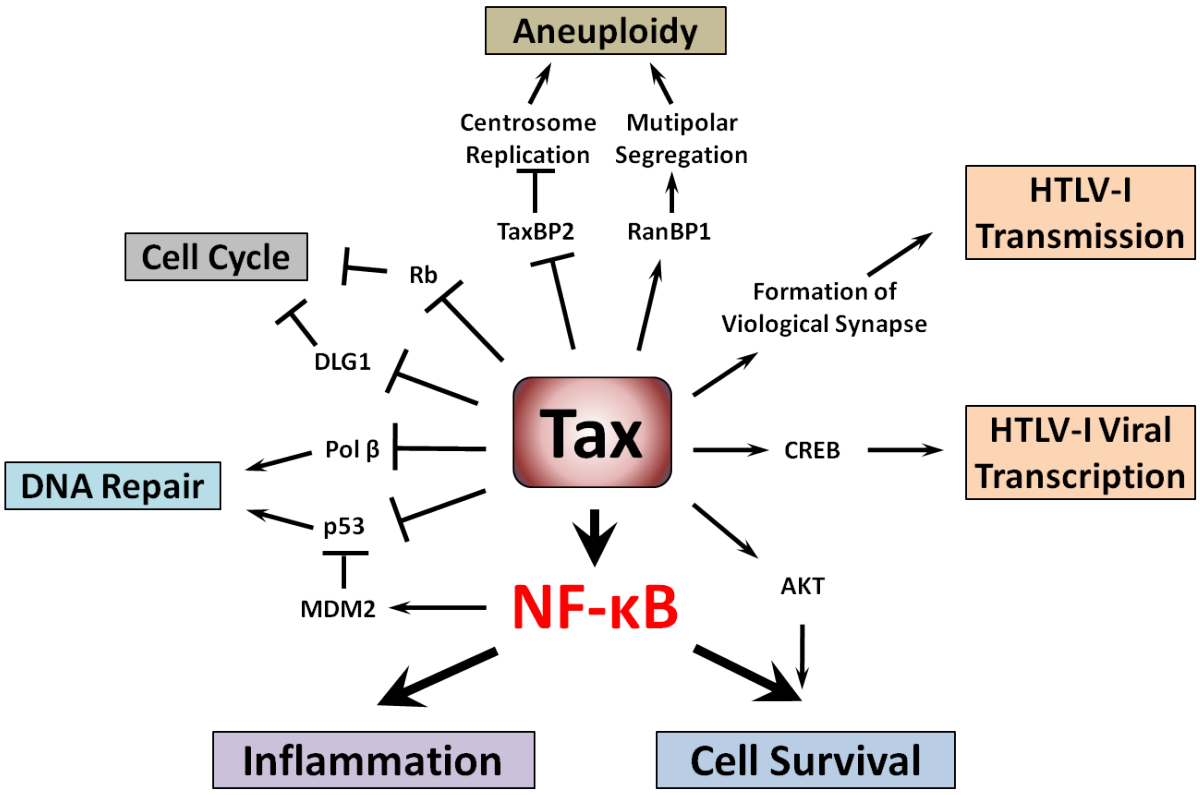

Figure 5. Schematic display of Tax functions.

Tax is an HTLV-I-specific oncoprotein and essential for HTLV-I-induced cell immortalization and transformation (Grassmann *et al.*, 1992; Grassmann *et al.*, 1989; Pozzatti *et al.*, 1990; Tanaka *et al.*, 1990). The functions of Tax include the promotion of viral transcription, activation of cell survival signaling, inactivation of intrinsic tumor suppressor, and contribution to DNA damage and chromosome instabilities (Kasai and Jeang, 2004; Matsuoka and Jeang, 2007; Yoshida, 2010). Tax has also been shown to benefit HTLV-I transmission by promoting viral synapse formation (Igakura *et al.*, 2003; Barnard *et al.*, 2005; Nejmeddine *et al.*, 2005).

1.1.3.1 Tax is required for HTLV-I-mediated cell transformation and tumorigenesis.

Tax not only transforms rodent fibroblasts but also immortalizes human primary T cells (Grassmann *et al.*, 1992; Grassmann *et al.*, 1989; Pozzatti *et al.*, 1990; Tanaka *et al.*, 1990). Compared to cells transformed by other cellular oncogenes, Tax-transformed cells have an apparently higher resistance to the induction of apoptosis, mainly through NF-κB activation and Bax repression (Brauweiler *et al.*, 1997; Copeland *et al.*, 1994; Fujita and Shiku, 1995; Sun and Yamaoka, 2005; Tsukahara *et al.*, 1999). In addition, Tax-transformed lymphoid cells and fibroblasts induce tumors when introduced into nude mice (Oka *et al.*, 1992; Pozzatti *et al.*, 1990). More importantly, the HTLV-I genome without Tax loses its original transformative ability, whereas Tax-transgenic mice develop spontaneous tumors, depending on the type of promoters used to drive Tax expression (Grossman *et al.*, 1995; Nerenberg *et al.*, 1987; Nerenberg, 1990; Peebles *et al.*, 1995; Yamaoka *et al.*, 1992). Indeed, Tax-immortalized lymphocytes *in vitro* and Tax-mediated T cell lymphomas in animals closely resemble the phenotypes of HTLV-I-transformed T cells and HTLV-I-induced ATL, respectively (Akagi *et al.*, 1995; Hasegawa *et al.*, 2006; Kwon *et al.*, 2005). Recent studies have demonstrated that HTLV-I

can also infect HSCs, supporting the existence of cancer stem cells (CSCs) and suggesting new mechanisms of ATL development involving CSCs (Banerjee *et al.*, 2010a; Banerjee *et al.*, 2008; Banerjee *et al.*, 2010b). Importantly, SCID mice inoculated with either HTLV-I or Tax infected HSCs develop CD4+ T cell lymphomas that recapitulate ATL in humans, further supporting the role of Tax in HTLV-I-mediated cell transformation and tumorigenesis (Banerjee *et al.*, 2010b).

1.1.3.2 Tax promotes HTLV-I viral transcription .

The functions of HTLV-I Tax include the promotion of viral transcription and the deregulation of multiple cellular signaling pathways (Figure 5). Cooperating with the cAMP-responsive element binding protein (CREB), Tax activates the expression of viral genes via an interaction with TRE, a conserved 21-bp repeat within the 5' LTR of HTLV-I (Adya *et al.*, 1994; Bantignies *et al.*, 1996; Fujisawa *et al.*, 1989; Jeang *et al.*, 1988; Kashanchi and Brady, 2005). The transcription activity of CREB is activated via a direct interaction between Tax and the bZIP (basic region leucine zipper) DNA-binding domains within CREB, resulting in elevated DNA binding activities (Kashanchi and Brady, 2005; Wagner and Green, 1993). Then the Tax-CREB complex facilitates the recruitment of coactivators like CBP and p300, resulting in the acetylation of histones and chromatin remodeling and permitting the activation of viral LTR, thereby promoting HTLV-I viral transcription (Bex and Gaynor, 1998; Georges *et al.*, 2002; Giebler *et al.*, 1997; Harrod *et al.*, 2000; Matsuoka and Jeang, 2007; Yoshida, 2010).

1.1.3.3 Tax deregulates multiple intracellular signal transductions.

Tax exerts its oncogenic properties largely through the deregulation of cellular transcription factors that are critical for cell growth and division, including NF-κB and AKT

(Hall and Fujii, 2005; Matsuoka and Jeang, 2007; Sun and Yamaoka, 2005). Those signals are required to quell the propensity of infected cells to enter apoptosis. The NF-κB signaling pathway is the prime target of Tax and is required for T cell immortalization by HTLV-I (Robek and Ratner, 1999). Studies have demonstrated that NF-κB inhibition suppresses the growth of Tax-transformed rodent fibroblasts, and that Tax-mediated NF-κB activation is responsible for the development of some skin diseases (Kwon *et al.*, 2005; Sun and Yamaoka, 2005; Yamaoka *et al.*, 1996).

In ATL cells, Tax induces the activation of NF-κB through a persistent degradation of IκB and the processing of p100, the canonical and non-canonical NF-κB activation pathways, respectively (Sun and Xiao, 2003; Xiao *et al.*, 2006). Tax interferes with the activation of NF-κB at multiple levels. In the cytoplasm, Tax binds to and recruits the IκB kinase (IKK) complex, an essential complex in NF-κB signaling, via its regulatory subunit IKKγ into specific compartments for IKK activation, resulting in the degradation of IκB and the subsequent nuclear translocation of NF-κB factors, including p65 (RelA) (Harhaj *et al.*, 2007; Xiao *et al.*, 2000; Xiao *et al.*, 2006). In the nucleus, Tax recruits p65 and other cellular transcriptional components into inter-chromatin sparkles to form transcriptional hot-spots termed as “Tax nuclear bodies” for full NF-κB transcriptional activation (Bex *et al.*, 1997; Semmes and Jeang, 1996).

Constitutive canonical NF-κB activation has been reported in various cancers. However, the aberrant expression of p52 in T cells is considered to be the key marker of HTLV-induced T cell transformation in ATL development, correlating with the observation that p52 production is tightly regulated in normal T cells, even when the cells are activated by T cell mitogens (Lanoix

et al., 1994; Xiao *et al.*, 2001a; Xiao *et al.*, 2006). The detailed mechanism by which Tax regulates NF-κB by Tax will be elucidated in the second part of this introduction.

Interestingly, the critical cytoplasmic and nuclear steps of NF-κB activation require two distinct post-translational modifications of Tax, namely ubiquitination and SUMOylation (Lamsoul *et al.*, 2005; Nasr *et al.*, 2006). In the cytosol, ubiquitination is crucial for Tax binding to IKK; while in the nucleus, SUMOylation of the same lysine residues in the Tax C-terminus is required for the formation of p65/p300-enriched Tax nuclear bodies and NF-κB transcriptional activation (Nasr *et al.*, 2006).

In addition to its role in NF-κB signaling, Tax interacts and activates phosphatidylinositol-3-kinase (PI3K) to stimulate the AKT signaling pathway. The AKT pathway contributes to both cell survival and proliferation and is associated with the apoptotic escape of HTLV-I-infected cells (Hall and Fujii, 2005; Jeong *et al.*, 2005; Peloponese and Jeang, 2006). In addition, the JNK pathway with the phosphorylation of c-Jun is regulated by HTLV-I Tax via interactions with small GTPases, including RhoA, Rac1 and Cdc42, which affect IL-2-independent growth and mediate the transition from the IL-2-dependent to IL-2-independent stage of HTLV-I-infected cells (Hall and Fujii, 2005; Jin *et al.*, 1997; Wu *et al.*, 2004; Xu *et al.*, 1996).

1.1.3.4 Tax contributes to the aneuploidy of ATL cells.

The appearance of flower cells which result from multipolar mitosis is a clinical marker of ATL and is associated with cell aneuploidy. Aneuploidy is widely prevalent in tumor cells and is responsible for DNA damage and chromosomal instability (Nigg, 2002; Pihan *et al.*, 2001;

Rasnick, 2002). Interestingly, abnormal centrosomes co-localize with Tax in ATL cells, suggesting that Tax contributes to aneuploidy (Ching *et al.*, 2006; Nitta *et al.*, 2006; Pumfery *et al.*, 2006). Evidence shows that Tax expression leads to centrosome over-duplication by regulating the centrosomal protein TAX1BP2 and/or Ran binding protein 1 (RanBP1) (Ching *et al.*, 2006; Peloponese *et al.*, 2005). Tax1BP2 is a centrosomal protein that normally blocks centrosome replication. The occupation of TAX1BP2 by Tax or the depletion of TAX1BP2 leads to hyper-amplification of centrosomes and causes aneuploidy (Ching *et al.*, 2006). Distinct from TAXBP2, Tax targets RanBP1 and facilitates multipolar cell segregation by impairing spindle poles (Peloponese *et al.*, 2005). Tax also binds to and inactivates MAD1, resulting in the loss of function of the mitotic spindle assembly checkpoint (SAC) (Jin *et al.*, 1998). Furthermore, abnormal chromosomal segregation due to the aberrant degradation of cyclin A, cyclin B1 and securin triggered by Tax is detected (Liu *et al.*, 2005). Taken together, these data lead to the belief that Tax favors premature mitosis and contributes to the aneuploidy of ATL cells (Kamihira *et al.*, 1994; Matsuoka and Jeang, 2007).

1.1.3.5 Tax inactivates tumor suppressors and provokes aberrant cell cycles and DNA repair.

Tax inactivates multiple tumor suppressor genes including Drosophila discs large tumor suppressor protein (DLG1), adenomatous polyposis complex (APC), Rb and p53 (Matsuoka and Jeang, 2007). Importantly, it was discovered that HTLV-I Tax has a wide interaction with PDZ domain-containing proteins via its C-terminal PDZ-binding motifs (PBMs). Generally, PDZ-domain-containing proteins associate with the cytoskeleton and serve to organize cell signaling assemblies to regulate cell growth, polarity and adhesion (Lee and Zheng; Sheng and Sala, 2001).

The protein hDLG1 protein is the human homologue to *Drosophila* DLG and is involved in the Wnt signaling pathway (Matsumine *et al.*, 1996; Woods and Bryant, 1991). DLG1 also plays a prominent role in regulating the cell-cycle phase transition by interacting with the C-terminus of the APC tumor suppressor. It has been reported that Tax physically binds to DLG1, subsequently inactivating DLG1 by leading to its hyper-phosphorylation and aberrant subcellular distribution; thus, Tax promotes cell proliferation by overcoming the G0/G1 transition (Aoyagi *et al.*, 2010; Suzuki *et al.*, 1999a). Notably, PDLIM2, which negatively regulates Tax, also belongs to the family of PDZ-domain-containing proteins.

In addition to its effects on DLG1, Tax contributes to cell-cycle transitions and overrides the G1/S transition by modulating the Rb protein. It induces the hyper-phosphorylation of Rb via interacting with Cdk4/Cdk6 or directly promoting the degradation of Rb protein (Fraedrich *et al.*, 2005; Haller *et al.*, 2002; Kehn *et al.*, 2005; Suzuki *et al.*, 1996). Tax also inactivates p53 via disrupting the interaction between p53 and p300/CBP by forming the Tax-p300-CBP complex, or inducing MDM2 expression to degrade p53 (Jeong *et al.*, 2004; Suzuki *et al.*, 1999b). Moreover, Tax represses DNA repair by down-regulating DNA polymerase β and inhibiting DNA topoisomerase I (Figure 5), so as to promote the mutation frequency (Jeang *et al.*, 1990; Suzuki *et al.*, 2000). In other words, Tax plays roles in modulating cell-cycle transitions, DNA repair, DNA damage responses and chromosomal instability. All of these functions are associated with HTLV-I-mediated pathogenesis.

1.1.3.6 Silencing of Tax facilitates the escape of HTLV-I-infected cells from the immune response, contributing to HTLV-I latency and persistent infection.

As described above, Tax is required to initiate HTLV-I-mediated leukemogenesis (Figure 3). However, Tax expression is largely suppressed or silenced during the late stage of ATL progression, indicating that Tax is no longer needed to maintain the transformed phenotype of ATL cells (Matsuoka and Jeang, 2007; Tamiya *et al.*, 1996; Yoshida, 2010). Because Tax is the target of CTLs, it is accepted that the suppression of Tax helps HTLV-I-infected cells to escape the immune response and contributes to HTLV-I latency (Bangham and Osame, 2005). Many mechanisms have been reported to be involved in Tax repression, including Tax negative regulators, genetic alteration, hyper-DNA methylation and deletion of the 5' LTR of the HTLV-I provirus (Aoyagi *et al.*, 2010; Furukawa *et al.*, 2001; Koiwa *et al.*, 2002; Matsuoka and Jeang, 2007; Takeda *et al.*, 2004; Taniguchi *et al.*, 2005).

To summarize, Tax is the primary oncogenic mediator for HTLV-I-induced cell immortalization and tumor formation. This viral protein provokes HTLV-I transcription as well as the stimulation of cell growth, which is associated with the deregulation of a number of cellular factors including NF-κB. Additionally, Tax contributes to tumorigenesis by inactivating tumor suppressor genes and/or inducing impaired cell checkpoint, DNA damage and chromosome instabilities.

1.1.4 Accessory genes from the HTLV-I pX region other than Tax

In addition to the importance of Tax, the accessory genes within the HTLV-I pX region that are generated from the alternatively spliced HTLV-I mRNA also contribute to HTLV-I viral

persistence. These genes include Rex, p12, p30 and HBZ (Matsuoka and Jeang, 2007; Nicot *et al.*, 2005).

As described before, Rex is required for the expression of viral structural gene products, including Gag, Pol and Env, which are crucial to HTLV-I replication and virus assembly (Figure 4). The cis-acting Rex responsive element (RRE) within the 3' LTR is the target of Rex. The occupation of Rex on RRE leads to the nuclear exportation of unspliced or singly spliced viral mRNA to the cytoplasm, which encodes the viral structural genes (Figure 4). Meanwhile, this occupation limits the production of spliced mRNA, which encodes Tax. In this way, Rex suppresses Tax production and contributes to a transient process of viral transcription and replication because Tax promotes HTLV-I transcription (Inoue *et al.*, 1987; Yoshida, 2010).

p12 is associated with host cell proliferation by affecting the threshold of NF-AT-mediated T cell activation and IL-2 production (Nicot *et al.*, 2001). In addition, p12 modulates cytoplasmic calcium and calcium-mediated cellular gene expression, including interleukin 6 signal transducer (IL6ST), members of the tumor necrosis factor (TNF) superfamily, adenosine receptor, TNF receptor-associated factor (TRAF), which play vital roles in intracellular signaling cascades (Ding *et al.*, 2001). It should be noted that p12 also inhibits the expression of major histocompatibility complex (MHC) class I by binding to heavy chains of free MHC class I proteins, resulting in the proteasomal degradation of newly synthesized MHC-I-hc (Johnson *et al.*, 2001). In this way, p12 protects infected cells from CTL recognition and maintains a persistent HTLV-I infection.

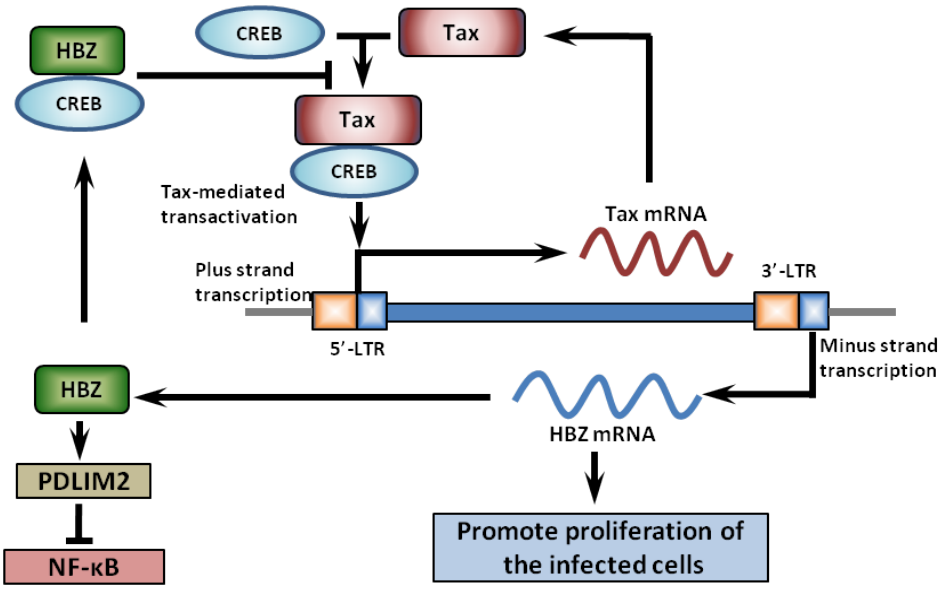

Figure 6. Functions of HBZ in HTLV-I-infected cells.

p30 plays a role as the negative regulator of HTLV-I and is related to the low-levels of viral antigens of HTLV-I-infected cells *in vivo* (Nicot *et al.*, 2004). By association with the doubly spliced Tax and Rex mRNA, p30 traps both Tax and Rex mRNAs (but not the structural genes' mRNAs) in the nucleus, resulting in the suppression of Tax and Rex expression at a post-transcriptional level. Moreover, p30 interacts with coactivators, including CBP and p300, disrupts the formation of Tax-p300-TRE complex and inhibits Tax-dependent LTR transactivation (Matsuoka and Jeang, 2007; Zhang *et al.*, 2000). Through those mechanisms, HTLV-I-infected cells present low levels of antigens on surfaces and escape the immune response.

HBZ, which is encoded by the minus strand of the provirus, serves as an important negative regulator of Tax (Figure 6). HBZ suppresses Tax mediated HTLV-I viral transcription by competing with interaction between Tax and CREB (Gaudray *et al.*, 2002; Matsuoka and Green, 2009). HBZ is constitutively expressed in the late stage of ATL, while Tax is largely

repressed at the same time, supporting the role of HBZ as a negative regulator of Tax. Moreover, recent studies showed that HBZ suppresses NF-κB activation via activation of a terminator of NF-κB named PDLIM2 (Figure 6, (Zhao *et al.*, 2009)), supporting the findings in this thesis that PDLIM2 negatively regulates Tax. Interestingly, HBZ mRNA, but not the protein, is sufficient to promote T cell proliferation, suggesting an oncogenic function and a crucial role in ATL development (Matsuoka and Green, 2009; Satou *et al.*, 2006).

In summary, Rex, HBZ and p30 serve as negative regulators of HTLV-I Tax and contribute to HTLV-I-mediated pathogenesis.

1.1.5 Negative regulators of HTLV-I Tax

Although the mechanisms by which HTLV-I Tax deregulates cellular signaling for oncogenesis have been extensively studied, how Tax itself is regulated by other cellular factors remains largely unknown. Knowledge about the negative regulators of HTLV-I Tax is still limited to the regulators from HTLV-I itself, including Rex, HBZ and p30, as described above (Basbous *et al.*, 2003; Inoue *et al.*, 1987; Nicot *et al.*, 2004; Nicot *et al.*, 2005).

Importantly, rather than inhibiting HTLV-I infection, all of these known negative regulators of Tax help the HTLV-I-infected cells to escape from the host's immune response (Figure 3), thereby contributing to persistent viral infection and HTLV-I-mediated pathogenesis. Considering the crucial role of Tax in the initiation of HTLV-I-induced leukemogenesis, it is necessary to discover the intrinsic negative regulators of Tax in the host cells, because they may be the primary defenders in determining the initial infection of HTLV-I and inhibiting HTLV-I pathogenesis. In this study, we defined PDLIM2 as the first intrinsic suppressor of Tax from the

host cells. PDLIM2 targets Tax for proteasomal degradation and suppresses Tax-mediated NF-κB activation, HTLV-I-LTR viral transcription and Tax mediated tumorigenesis. Unlike other PDZ-domain-containing proteins, PDLIM2 binds to Tax via its internal disordered sequences other than the PDZ domain, while multiple distinct sequences within Tax are responsible for the interaction with PDLIM2. Chapter 2 of this dissertation will focus on elucidating the mechanism of Tax regulation by PDLIM2, and Chapter 3 will explore the molecular determinants of PDLIM2 in Tax regulation.

1.1.6 Therapeutic approaches and the prevention of HTLV-I infection

Thirty years after the initial clinical determination of ATL as an HTLV-I-related disease, therapeutic approaches are still lacking. This is mainly because HTLV-I-transformed cells are highly resistant to the induction of apoptosis (Grassmann *et al.*, 2005; Matsuoka and Jeang, 2007; Taylor and Matsuoka, 2005; Uozumi, 2010). There is still no beneficial treatment for ATL other than allogeneic hematopoietic stem cell transplantation.

1.1.6.1 Chemotherapy

Chemotherapy, including cyclophosphamide, adriamycin, vincristine and prednisolone (CHOP), is the primary standard treatment for ATL. The intensive chemotherapy protocol LSG-15, using VCAP (vincristine, cyclophosphamide, doxorubicin and prednisone), AMP (doxorubicin, ranimustine and prednisone) and VECP (vindesine, etoposide, carboplatin and prednisone) with G-CSF (granulocyte colony-stimulating factor) support, is reported to have improved efficacy (Taylor and Matsuoka, 2005; Uozumi, 2010; Yamada *et al.*, 2001). Other approaches, such as targeting the cell differentiation markers like CD25 via the injection of

monoclonal CD25 antibody, a combination treatment with interferon and zidovudine, and the usage of topoisomerase inhibitors, including CPT11 and MST16 also yield partial or complete remission in some specific individual cases (Hermine *et al.*, 1995; Makino *et al.*, 1994; Ohno *et al.*, 1993; Uozumi, 2010). Unfortunately, the general benefits are poor.

1.1.6.2 Stem cell transplantation

Allogeneic hematopoietic stem cell transplantation remains the only beneficial treatment for ATL. Through this method, the HTLV-I proviral load can be reduced to an undetectable level in many cases (Obama *et al.*, 1999; Tajima *et al.*, 2000; Uozumi, 2010). However, this approach is restricted to aggressive ATL treatment because of the high incidence of fatal toxic effects and transplantation-related disease (Kami *et al.*, 2003; Utsunomiya *et al.*, 2001; Yonekura *et al.*, 2008). The real curative effect is still under investigation. Therefore, finding new targets and discovering novel drugs for HTLV-I-related disease are still necessary. Targeting NF-κB for drug discovery is the new hope for ATL treatment.

1.1.6.3 New targets for ATL treatment

As described before, ATL is an NF-κB related disease. Considering the essential role of Tax in HTLV-I-mediated pathogenesis, current drug discovery efforts and testing against ATL mainly focus on targeting anti-Tax immunization and Tax-related signaling pathways like NF-κB and AKT. A numbers of tested drugs for ATL treatment work either directly or indirectly through the regulation of NF-κB (Uozumi, 2010). The proteasome inhibitor PS-341, which inhibits NF-κB by efficiently blocking IκB degradation, inhibits the growth of ATL cells *in vitro* and ATL cell induced xenografts *in vivo* (Satou *et al.*, 2004). Previous data from our group

showed that treatment with geldanamycin (GA), an Hsp90 inhibitor, can inhibit the NF-κB signaling pathway and lead to the apoptosis of HTLV-I-transformed T cells (Yan *et al.*, 2007). Additional, evidence has shown that Tax-expressing cells undergo apoptosis after treatment with PI3K inhibitors to prevent Akt phosphorylation (Ikezoe *et al.*, 2007). All of these inhibitors give insight into therapeutic approaches and new hopes for this incurable disease.

The development of cancer involves the epigenetic repression of tumor suppressor genes. In Chapter 4 of this thesis, we report that the treatment of DNMT inhibitors induces the death of HTLV-I-transformed T cells and ATL cells in association with the reactivation of PDLIM2, an NF-κB suppressor. Although the DNMT inhibitor 5-aza-dC may not only affect PDLIM2 reactivation, the clinical trial of this phase-III drug shows potential therapeutic capacity for ATL treatment.

1.1.6.4 Prevention of HTLV-I infection

Although ATL treatment remains poor, there have been impressive achievements in ATL prevention. Because the mechanisms of HTLV-I transmission are well-studied, the spread of HTLV-I has been reduced by 80% by avoiding breastfeeding in HTLV-I-carrying mothers in Japan (Taylor and Matsuoka, 2005). However, due to the cost of formula and the potential risk of infant mortality from diarrheal diseases related to bottle feeding, this efficient method may not translate to execution in many countries with a high prevalence of HTLV-I (Hanchard, 1999). Nowadays, vaccinations with HTLV-I Tax-targeted vaccines in carriers with high proviral loads and low HTLV CTL responses are highly recommended, and improvements have been achieved

(Bangham and Osame, 2005). The extensive research on HTLV-I has provided significant contributions to the current longer latency and lower risk of HTLV-I infection.

1.2 THE NF-KB SIGNALING PATHWAY AND ITS FUNCTIONS IN TUMORIGENESIS

Mammals have an organized system to defend against various infectious agents that is mediated by the early reaction of innate immunity and the later responses of adaptive immunity. The innate immune system recognizes pathogen-derived substances and its activation results in the expression of numerous antimicrobial molecules. In contrast, the adaptive immune system leads to DNA rearrangement in somatic cell and generates antigen-specific receptors, or antibodies. Both steps are initiated by the recognition of pathogens, which then induce the activation of intracellular signaling cascades (Janeway, 2005). Among the activated nuclear transcription factors, NF-κB plays a central role as it regulates and influences various biological processes, including inflammation, the immune response, cell survival and tumorigenesis (Ghosh *et al.*, 1998; Hayden and Ghosh, 2008; Silverman and Maniatis, 2001).

NF-κB was firstly identified as a regulator of the immunoglobulin κ light chain gene in B cells 25 years ago (Sen and Baltimore, 1986). Later studies confirmed the conservation and ubiquities of NF-κB signaling from flies to humans (Silverman and Maniatis, 2001). Nowadays, it has been demonstrated that NF-κB is not only a central mediator of immune responses, but also a regulator in various pathogeneses, particularly oncogenesis (Sun and Xiao, 2003; Xiao *et al.*, 2006). As described in the former section, HTLV-I-induced ATL is an NF-κB-related disease, and most currently tested drugs for ATL target NF-κB signaling, either directly or indirectly. Importantly, HTLV-I Tax exerts its oncogenic role largely through the deregulation of cellular

transcription factors that are critical for cell growth and division, mainly through NF-κB (Matsuoka and Jeang, 2007; Yoshida, 2010).

1.2.1 NF-κB members

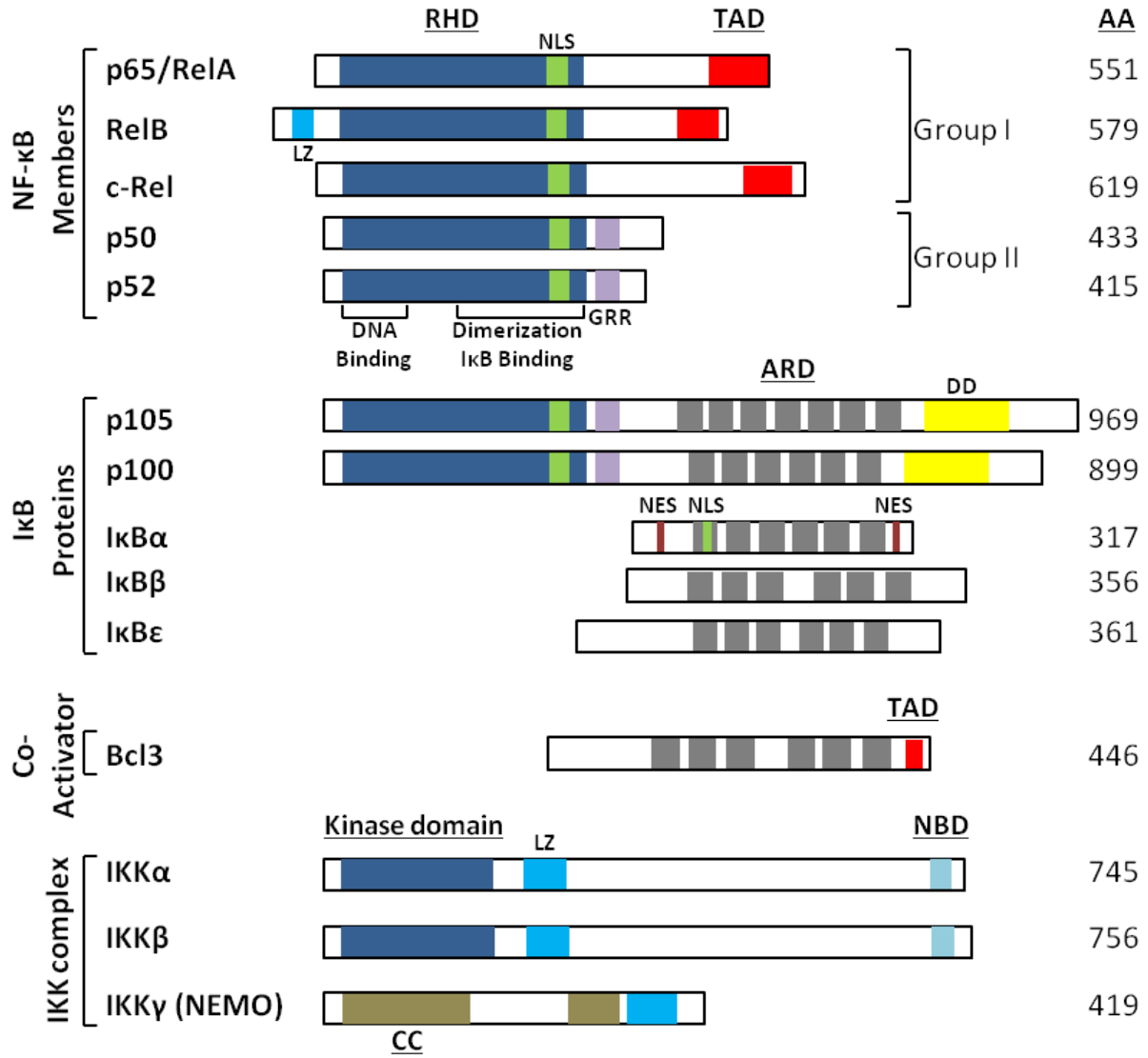

Figure 7. The NF-κB, IκB and IKK protein families.

Members of the NF-κB, IκB, and IKK proteins are shown. The number of amino acids in each human protein is presented on the right. RHD, Rel homology domain; TAD, transactivation domain; LZ, leucine zipper domain; GRR, glycine-rich region; CC, coiled-coil domain; NBD NEMO-binding domain; DD, death domain; NLS, nuclear localization sequences; NES, nuclear export sequences.

There are five NF-κB family members in mammals: p65 (RelA), RelB, c-Rel, NFκB1 (p105/p50) and NFκB2 (p100/p52), encoded by *RELA*, *RELB*, *REL*, *NFKB1* and *NFKB2*, respectively (Hayden and Ghosh, 2008; Xiao *et al.*, 2006). All those members share an N-terminal Rel homology domain, which is responsible for binding to κB sites, dimerization and NF-κB nuclear translocation (Figure 7). Based on the different structures and functions of their C-terminals as well as the different synthesis modes, the five NF-κB members can be classified into two groups. RelA, RelB and c-Rel proteins comprise the group containing the C-terminal transcription activation domain (TAD), which is necessary for the positive regulation of NF-κB target gene expression (Figure 7). Notably, NFκB1/p50 and NFκB2/p52 are synthesized as large precursor forms, p105 and p100, respectively (Figure 7). Their C-termini can be selectively degraded via the ubiquitin-proteasome system. The remaining N-terminal regions, which are p50 and p52, could dimerize with other NF-κB family members due to the existence of an RHD domain.

NF-κB members form numerous homo- and hetero-dimers that are associated with specific biological responses in regard of their ability to regulate the transcription of different target genes. Lacking the TAD domain, NFκB1/p50 and NFκB2/p52 repress transcription unless forming heterodimers with other NF-κB members that have TADs. This repression feature has been used to discover the transcriptional competitors of NF-κB dimers by measuring κB-driven luciferase reporter gene activities. In this system, p50 or p52 homodimers constitutively bind to κB sites with suppressed luciferase activities, while the presence of transcriptional competitors results in transcriptional activation. Additionally, p50 or p52 homodimers can become transcriptional activators when associated with the BCL3 co-activator family.

NF-κB dimers are usually trapped in the cytosol through interaction with members of IκB proteins, including IκBα, IκBβ, IκBγ and IκBε. The IκBs are defined by the presence of five to seven ankyrin repeat domains (ARD), which interact with the RHDs, mask the nuclear localization sequences (NLS), and prevent the nuclear translocation of NF-κB dimers (Figure 7). Thus, the basic scheme of NF-κB signaling requires the degradation of IκB proteins. Importantly, the precursors of NFκB1/p50 and NFκB2/p52, p105 and p100, also contain this ARD at their C-termini so that both of them can function as IκB like inhibitors (Figure 7). Considering that p105 processing is a constitutive event whereas p100 processing is tightly controlled and highly inducible, the processing of p100 also leads to NF-κB activation because it will liberate p100 containing dimers and drive a transcriptional response (Xiao *et al.*, 2006). Because of the distinct effects and target genes of IκB degradation and p100 processing in the regulation of NF-κB activation, they are characterized as canonical (classic) or non-canonical (non-classic) NF-κB pathways (Figure 8).

1.2.2 Canonical NF-κB pathway

In the canonical NF-κB signaling pathway, the essential step is the phosphorylation and subsequent degradation of IκB (Figure 8). Upon stimulation by proinflammatory cytokines, mitogens, antigens and DNA damage, the activated IKK complex acts predominantly via IKKβ to catalyze the phosphorylation of IκB at specific serine residues (S32 & S36) (Hacker and Karin, 2006). The phosphorylated IκB is then sequestered by the β-TrCP-SCF complex, resulting in its poly-ubiquitination and degradation by the 26S proteasome, allowing the translocation of bound NF-κB dimers into the nucleus and inducing the expression of target genes.

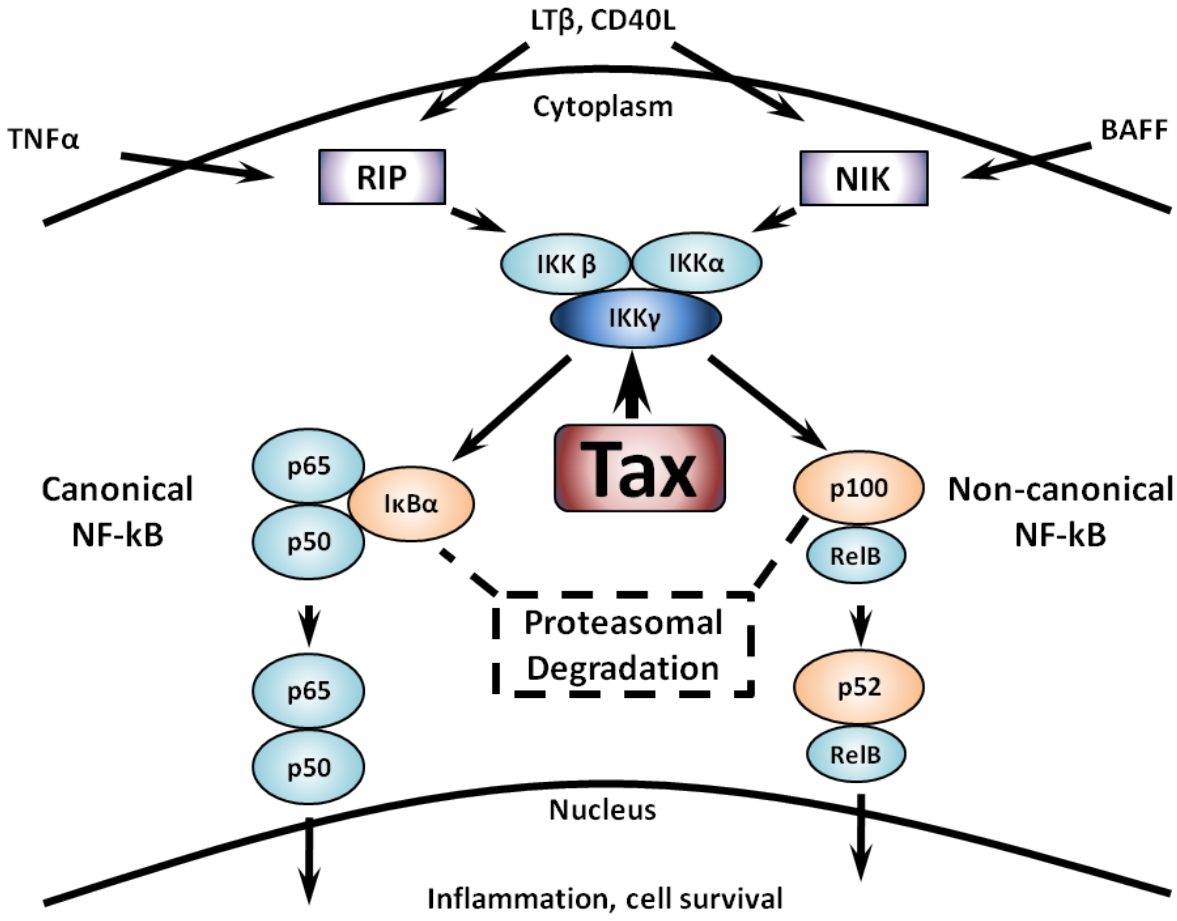

Figure 8. Canonical and non-canonical NF-κB signaling pathways.

The p65-containing NF-κB dimers, especially the p65/p50 heterodimer, are the prototypic transactivator in canonical NF-κB pathway. In addition to IKK-mediated IκB degradation, modifications including phosphorylation and acetylation of p65 are indispensable in canonical NF-κB activation. p65 can be phosphorylated directly or indirectly by many kinases such as IKK, NIK, CK2, PKA, PKCζ, PI3K, GSK3β, RSK1, TBK1, AKT and p38 (Viatour *et al.*, 2005). Phosphorylated p65 is necessary to the transcriptional competence of p65-containing NF-κB dimers, as it induces conformational changes that facilitate DNA binding and recruit transcriptional coactivators including CBP-p300. CBP-p300 contributes by inducing the

acetylation of p65. Interestingly, especially at Lysine 310, the phosphorylation and acetylation of p65 actually share the same residue (Greene and Chen, 2004). It has been reported that the phosphorylation prominently recruits CBP-p300 and allows the subsequent acetylation, resulting in p65-mediated activation of NF-κB. In addition, phosphorylation stabilizes p65 and inhibits the binding to IκBα via Pin1-dependent prolyl isomerization (Ryo *et al.*, 2003). The large number of kinases involves in p65 phosphorylation, also correlates the fact that the activation of distinct kinases is specific to distinct cell types or stimuli that activate NF-κB.

1.2.3 Non-canonical NF-κB pathway

The large precursors, p105 and p100, function as IκB-like inhibitors, while the processed forms, p50 and p52, are partners of other NF-κB family members as well as Bcl3 co-activator family members. The processing of p50 and p52 produces specific functional NF-κB complexes. Notably, the processing of p105 to p50 is constitutive, while p52 production is tightly regulated (Xiao *et al.*, 2001a; Xiao *et al.*, 2001b; Xiao *et al.*, 2006). In most cell types, p50 dimerizes with p65 and forms the prototypical NF-κB dimer. By contrast, the NF-κB activation by p52-containing dimers results from the inducible p100 processing, known as the non-canonical NF-κB pathway (Figure 8).

Distinct from the broad biological significance of the canonical pathway in inflammation, the non-canonical pathway is mainly involved in B cell maturation and lymphoid tissue development (Caamano *et al.*, 1998; Franzoso *et al.*, 1998). *NFKB2*-knockout mice present defects in B-cell function and impaired formation of peripheral lymphoid organs. Evidence from mice studies indicates the essential roles of NIK and IKKα in non-canonical NF-κB signaling

(Senftleben *et al.*, 2001; Xiao *et al.*, 2001a, 2001b). After the stimulation, NIK is stimulated by its upstream receptors like LTβR and subsequently activates IKKα. Then, the activated IKKα is recruited into the p100 complex through p100's C-terminal serine 866 (Xiao *et al.*, 2004). This serine induces proteolytic processing of p100 triggered by site-specific phosphorylation, poly-ubiquitination mediated by β-TrCP and selective degradation by the 26S proteasome (Fong and Sun, 2002; Xiao *et al.*, 2001a; Xiao *et al.*, 2006). As opposed to the complete degradation of IκB protein in the canonical NF-κB activation as described above, this poly-ubiquitination event on p100 only results in the selective degradation of its inhibitory C-terminus. Once the ARD inhibitory region is degraded, the N-terminus of p100 (p52) is released and the p52-containing NF-κB dimer becomes functional. As RelB is predominantly associated with p100, the activation of the non-canonical pathway leads to the nuclear translocation of the RelB-p52 heterodimer (Solan *et al.*, 2002).

The non-canonical NF-κB pathway is strictly dependent on the activity of NF-κB-inducing kinase (NIK) and IKKα, but it is independent of IKKβ and IKKγ, which are two central regulators of the canonical NF-κB pathway, and are tightly controlled under normal conditions (Senftleben *et al.*, 2001; Xiao *et al.*, 2001b). Consistently, under physiological conditions, the non-canonical pathway can only be induced by certain stimuli including B-cell activating factor (BAFF), CD40 ligand, Lymphotoxin beta (LTβ), TNF-like weak inducer of apoptosis (TWEAK) and the receptor activator of nuclear factor kappa B ligand (RANKL) (Claudio *et al.*, 2002; Coope *et al.*, 2002; Kayagaki *et al.*, 2002; Novack *et al.*, 2003; Saitoh *et al.*, 2003).

Importantly, the non-canonical NF-κB pathway can be hijacked by multiple viral oncoproteins, such as Tax, which is encoded by HTLV-I (Xiao *et al.*, 2001a). It is the first

known pathogenic inducer to constantly activate the p100 processing. Rather than requiring activated NIK, Tax induces p100 processing via interacting with IKKγ and targeting IKKα to p100 to trigger p100 phosphorylation, poly-ubiquitination and the production of p52. Importantly, the overproduction of p52 is considered as a hallmark of HTLV-I infection and transformation (Sun and Yamaoka, 2005; Xiao *et al.*, 2006). Results have shown that HTLV-I-infected cells exhibit constitutive p100 processing as well as the activation of NF-κB, while such processing is tightly controlled even in mitogen-activated T cells (Xiao *et al.*, 2001a).

1.2.4 IKK complex

The IKK complex plays a crucial role in both canonical and non-canonical NF-κB pathways. It typically consists of the IKKα and IKKβ catalytic subunits and the IKKγ (NF-κB essential modulator, NEMO) regulatory subunit. IKK is a multi-protein complex with a high molecular weight upon gel filtration (Agou *et al.*, 2004). Studies have indicated that CDC37, ELK and Hsp90 are also components of IKK (Chen *et al.*, 2002; Ducut Sigala *et al.*, 2004; Hayden and Ghosh, 2008). Although it is accepted that the core IKK complex most likely consists of only IKKα/IKKβ/IKKγ, those findings favor a model in which the IKK complex is dynamically formed with different components in response to different stimuli or depending on different cell types.

IKKγ is essential to NF-κB activation through the assembly of the IKK complex. IKKγ-deficient cells present a dramatic loss of NF-κB activation and mice without IKKγ have an embryonic lethal phenotype with aberrant hepatocyte apoptosis (Schmidt-Supprian *et al.*, 2000). IKKγ recruits the NEMO binding domains within the C-terminals of IKKα and IKKβ through

the amino acids 47-80, a coiled-coil motif (Drew *et al.*, 2007; Marienfeld *et al.*, 2006). Importantly, the assembly function of IKKγ is indispensable in the activation of IKK because the C-terminal mutants responsible for IKKγ in assembling the IKK complex fail to trigger IKK activation (Makris *et al.*, 2002). Given that IKKγ contains multiple distinct domains, IKKγ actually serves as a mediator linking the NF-κB-inducing signals to the activation of IKK, including the recruitment of upstream activators or viral proteins like Tax, resulting in the activation of NF-κB (Xiao *et al.*, 2000).

IKKα and IKKβ are structurally and biochemically similar to each other, sharing 52% overall sequence identity and 65% similarity in the catalytic domain; however, they have distinct biological functions (Hayden and Ghosh, 2008). IKKβ is predominantly responsible for most stimuli-induced IκB phosphorylation and degradation, as further supported by the observations of similar phenotypes between IKKβ- and p65- knockout mice (Beg *et al.*, 1995; Gerondakis *et al.*, 2006; Tanaka *et al.*, 1999).

In contrast, although IKKα activation can be induced by remarkably few stimuli including the receptor activator of NF-κB (RANK) ligand and the viral protein called Fas-associated death domain-like IL-1b converting enzyme inhibitory protein (vFLIP), it has relatively broad additional functions aside from those of IKKβ. Although it is reported that IKKα contributes to canonical NF-κB activation by inducing the phosphorylation of p65 and histone H3 (Yamamoto *et al.*, 2003), IKKα is largely expendable in canonical NF-κB activation because IKKα-null embryos appear to be phenotypically normal in IκBα degradation, NF-κB nuclear translocation and NF-κB DNA binding activity (Hu *et al.*, 1999; Takeda *et al.*, 1999). Further studies have indicated that the crucial role of IKKα in the non-canonical NF-κB signaling

pathway is the triggering of p100 phosphorylation (Xiao *et al.*, 2001a). In addition, IKKα is involved in the formation of the epidermis and induces keratinocyte differentiation via an NF-κB-independent mechanism (Hu *et al.*, 2001). Recently, it was reported that IKKα is involved in the development of prostate cancer, especially in the metastatic phase (Luo *et al.*, 2007). In that case, IKKα was activated and accumulated in the nuclei, resulting in a reduction of the metastasis inhibitor. Although such suppressed metastasis by IKKα seems to be NF-κB-independent, the detailed mechanisms are still under investigation (Luo *et al.*, 2007).

The leucine zipper domains within the N-termini of IKKα and IKKβ are required for their dimerization and kinase activity (Hayden and Ghosh, 2008; Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Xiao *et al.*, 2006; Zandi *et al.*, 1997). An *in vitro* kinase assay showed that the heterodimer form has a higher catalytic activity than either homodimer. IKKα and IKKβ consistently prefer the formation of heterodimers *in vivo* (Huynh *et al.*, 2000). However, under some circumstances, a homodimer of IKKβ is also observed *in vivo* (McKenzie *et al.*, 2000). Of note, although there is little evidence for the existence of either homodimer, it is believed that the IKKα homodimer is formed *in vivo* because IKKβ and IKKγ are independent of non-canonical NF-κB activation (Hayden and Ghosh, 2008; Senftleben *et al.*, 2001). Importantly, the HTLV-I viral oncoprotein Tax only recruits IKKα, but not IKKβ, into p100 for p100 phosphorylation and subsequent processing to p52 (Xiao *et al.*, 2001a).

1.2.5 NF-κB members in cancer

Cancer is a hyper-proliferative disorder that involves uncontrolled cell growth, migration, angiogenesis, invasion and metastasis (Grivennikov *et al.*, 2010). NF-κB participates in all those

processes and contributes to tumorigenesis by inducing many target genes related to anti-apoptosis, including Bcl-2, A20, cellular inhibitors of apoptosis (cIAPs) and cellular FLICE inhibitory protein (cFLIP). In addition, it also antagonizes p53 to prevent the death of cells that have generic alterations, facilitating neoplasm formation (Webster and Perkins, 1999). NF-κB mainly stimulates cell proliferation by: 1) directly up-regulating cell-cycle-related genes like cyclin D1 (CCD1), inducing hyper-phosphorylation of retinoblastoma (Rb) and promoting the cell-cycle transition from G1 to S phase; 2) indirectly inducing the expression of cytokines and cellular growth factors including IL-2, resulting in persistent activation of NF-κB and other proliferation-related signaling pathways (Baldwin, 2001).

Numerous studies have indicated that aberrant NF-κB activation is associated with advanced stages of oncogenesis, providing evidence for some contribution from NF-κB in tumor progression. Consistent with this evidence, many metastasis- and angiogenesis-related genes are also targets of NF-κB, such as chemokine IL-8, vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX2), intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1/CD106), inducible NO-synthase (iNOS) and cell-surface protease matrix metalloproteinase 9 (MMP9) (Pahl, 1999; Sun and Xiao, 2003). Tumor cells with constitutive NF-κB activation exhibit significantly up-regulated expression of these genes. The NF-κB members are the direct effectors in NF-κB regulation. It is necessary to understand the genetic alterations to NF-κB members and their outcomes.

A study on the v-Rel oncoprotein of the avian Rev-T retrovirus, which is a Rel homology member and induces fatal lymphomas and leukemias, provides the evidence linking NF-κB to oncogenesis (Gilmore, 1999). Consistently, v-Rel exhibits increased nuclear translocation and

constitutive activity in transcription, thereby leading to its transformational activity. Similar to the case for v-Rel, the over-expression of c-Rel can transform primary chicken lymphoid cells and primary avian fibroblasts, though at a lower frequency than v-Rel (Rayet and Gelinas, 1999).

Turning specifically to human cancer, genetic alterations leading to high expression and/or constitutive activity of NF-κB members have been linked to certain cancers. As an example, amplification of the *REL* gene is found in 23% of diffuse lymphomas with a large cell component (DLLC) and 50% of non-small cell lung carcinomas (NSCLC), as well as in primary mediastinal B-cell lymphomas and in certain follicular large lymphomas (Rayet and Gelinas, 1999). c-Rel expression increases four- to thirty-five-fold due to amplification of the *REL* gene (≥4 copies). Although it is unclear whether there is a certain association between increased c-Rel expression and tumorigenesis in these cases, it may contribute to progression of these diseases when considering the transformative ability of c-Rel as described above. Additionally, the rearrangement of the *REL* gene to create hybrid proteins, which is not as common as *REL* gene amplification in tumors, was observed in some follicular lymphomas and DLLCs (Rayet and Gelinas, 1999).

In contrast to the amplification of the c-rel gene, the genetic alteration of *NFKB2* by chromosome recombination or deletion results in the production of truncated p100 without full C-terminal inhibitory region (Rayet and Gelinas, 1999). The loss of the C-terminus of p100 determines the function of these truncated mutants. The outcomes of these truncations include a loss of function as an NF-κB inhibitor by deletion of the ARDs, a gain of function as a transactivator to translocate into the nucleus even with partial ARDs remaining and a gain of function to induce constitutive p52 production by losing the processing-inhibitory domain (PID)

so as to induce persistent non-canonical NF-κB activation (Qing *et al.*, 2005a). Consistently, these truncated p100 mutants present oncogenic ability *in vitro*, suggesting that the tumors associated with *NFKB2* gene arrangements indeed result from those mutations (Ciana *et al.*, 1997). In addition, the rearrangements of *NFKB2* have been associated with a variety of lymphomas including cutaneous B and T cell lymphomas, chronic lymphocytic leukemias (CLL) and multiple myeloma (Rayet and Gelinas, 1999; Sun and Xiao, 2003; Xiao *et al.*, 2006).

Additionally, unpublished data from our lab support the hypothesis that both the p100 precursor and the processed subunit p52 are required for tumor growth. The results show that REF cells stably expressing LB40 (the p100 mutant with a constitutive processing ability) generate xenografts in SCID mice (100%), while the mice injected with cells expressing the LB40 mutant (little processing) and p52 developed fewer (33%) or no tumors, respectively. In other words, the tumor growth is roughly correlated with the processing ability. Although confirmation of this will require more evidence, this study provides insights into the pertinent roles of both p100 and p52 in tumorigenesis.

In contrast to *REL* and *NFKB2* genes, genetic alterations to *RELA* and *NFKB1* are rare in human cancer, and no alteration of the *RELB* locus has been reported (Rayet and Gelinas, 1999). Chromosomal aberrations involving *RELA* are only found in B cell non-Hodgkin's lymphomas and multiple myelomas, and gene rearrangement of *NFKB1* only occurs in certain acute lymphoblastic leukemias (Houldsworth *et al.*, 1996; Lai *et al.*, 1995). However, elevated expression levels of p65 and p50 have been found in many different types of tumors, including thyroid carcinoma and 80% of non-small cell lung carcinomas (NSCLC) (Bours *et al.*, 1994; Visconti *et al.*, 1997), indicating the involvement of NF-κB regulators in cancer. Moreover, this

evidence not only indicates the predominant functions of p65 and p50 in NF-κB activation, but also suggests that constitutive activations by p65- and/or p50-containing complexes are required for the aberrant survival and growth of tumor cells.

1.2.6 Balance between positive and negative regulators in NF-κB signaling

The basic scheme of NF-κB signaling depends on the counterbalance between its activators and inhibitors. Generally, NF-κB activators are oncogenic, while NF-κB inhibitors exhibit tumor-suppressing functions (Figure 9). Nowadays, most efforts on the regulation of NF-κB focus on mechanisms that involve the IκB protein and signaling intermediates (Hayden and Ghosh, 2004, 2008). Considering that tumor progression is always associated with constitutive NF-κB activation, targeting NF-κB for tumor suppression actually shuts off NF-κB by repressing NF-κB activators and/or stimulating NF-κB inhibitors.

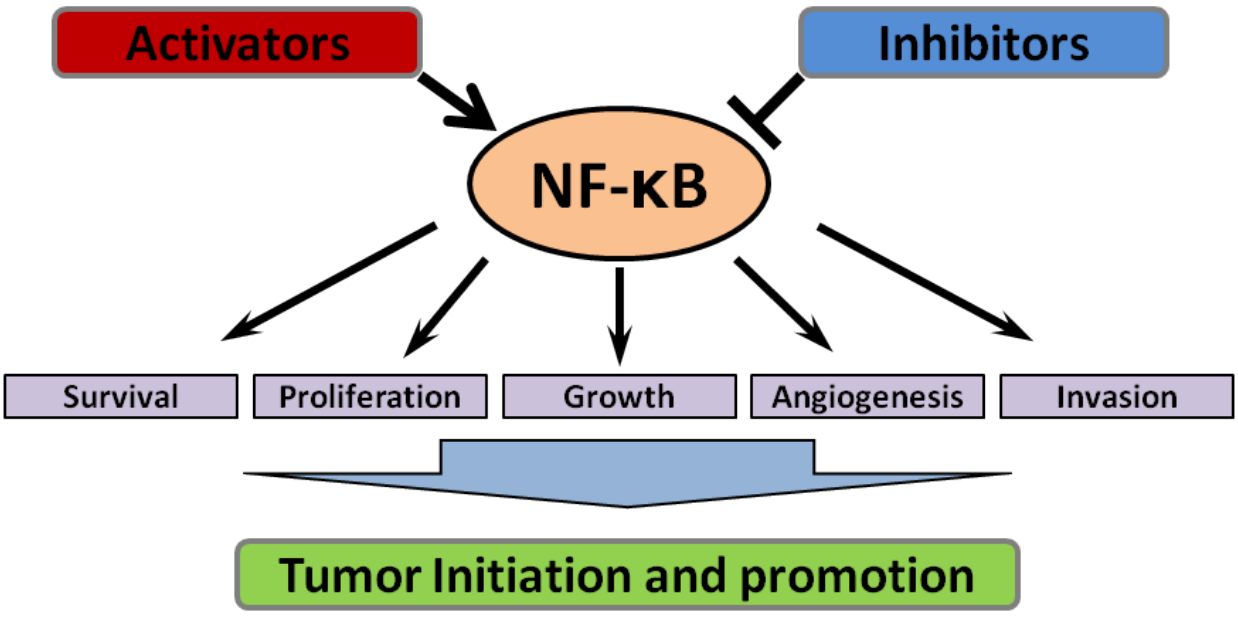

Figure 9. The basic scheme of NF-κB in tumorigenesis.

IκB family members, particularly IκBα, are the principle inhibitors of NF-κB signaling and act by sustaining NF-κB dimers in the cytosol. It is well accepted that IκB re-synthesis is the primary mechanism for shutting off NF-κB. Additional mechanisms exist: IKKα is involved in turning off inflammatory responses by accelerating the turnover of RelA and c-Rel in microphages (Lawrence *et al.*, 2005). Additionally, negative feedback mechanisms driven by the deubiquitinases A20 and CYLD have been demonstrated to suppress NF-κB by modulating the modifications to upstream signaling components like TRAF (Chen *et al.*, 2006; Hacker and Karin, 2006).

Another mechanism is negative regulation of the NF-κB dimer itself. It is reported that the copper metabolism MURRI-domain-containing 1 (COMMD1) protein is responsive for cleaning out the promoter-bound p65 complex with the involvement of suppressor of cytokine signaling 1 (SOCS1), which is sufficient to suppress NF-κB transcriptional activity in LPS mediated signaling (Kinjyo *et al.*, 2002; Maine *et al.*, 2007; Nakagawa *et al.*, 2002; Saccani *et al.*, 2004). Of note, PDLIM2 is reported to function as a novel ubiquitin E3 ligase of p65, which directly triggers the poly-ubiquitination and proteasomal degradation of nuclear p65, subsequently shutting off NF-κB (Tanaka *et al.*, 2007). The studies in this thesis will characterize the function of PDLIM2 as a negative regulator of the HTLV-I oncoprotein Tax, which serves as an NF-κB activator.

1.2.7 NF-κB regulators in cancer

NF-κB signaling depends on both NF-κB members and NF-κB regulators, including inhibitors, activators and co-activators. Other than genetic alterations on NF-κB members, cancer development is associated with the function of NF-κB regulators.

Defective IκBα activity has been detected in human tumors like hematopoietic tumors (Rayet and Gelinas, 1999; Sun and Xiao, 2003), and a subset of Hodgkin's lymphomas involves mutations or deletions of the *IκBα* gene, generating non-functional or unstable IκBα mutants (Cabannes *et al.*, 1999; Emmerich *et al.*, 1999; Jungnickel *et al.*, 2000).

In addition, many of kinases contribute to the oncogenesis associated with elevated NF-κB activity. As a matter of fact, most known oncoprotein kinases have been linked to NF-κB activity (Sun and Xiao, 2003). As an example, Tpl2 (MAP3K8) has been identified as a target for provirus integration in Moloney murine leukemia virus (MMLV)-induced T cell lymphomas, and the truncated Tpl2 mutant leads to T cell transformation and the development of lymphomas in transgenic mice (Ceci *et al.*, 1997; Makris *et al.*, 1993). Studies indicate that Tpl2 is associated with p105 precursor *in vivo* and stimulates the IKK complex for NF-κB activation upon stimulation (Lin *et al.*, 1999; Waterfield *et al.*, 2003). Moreover, in EBV-associated malignancies, the over-expression of Tpl2 contributes to LMP1-induced NF-κB activation and the expression of the angiogenic mediator COX-2 (Eliopoulos *et al.*, 2002).

Interestingly, in addition to the above regulators, the co-activator Bcl3 performs unique functions (Figure 7). Bcl3 was initially characterized as an inhibitor of NF-κB according to its C-terminal ARD (Hayden and Ghosh, 2008; Wulczyn *et al.*, 1992). However, unlike other IκB

members, Bcl-3 functions as a transactivator to remove inactive p50 homodimers from DNA or as a coactivator by interacting with p52 homodimers (Bours *et al.*, 1993; Fujita *et al.*, 1993; Nolan *et al.*, 1993). Additionally, Bcl3-deficient mice exhibit a partial loss of germinal centers and are insensitive to certain stimuli (Schwarz *et al.*, 1997). The over-expression of Bcl3 in B cells leads to a lympho-proliferative disorder through the accumulation of mature B cells in Bcl3-transgeneic mice (Ong *et al.*, 1998).

To summarize, NF-κB is evolutionarily conserved in the immune system and has been linked to inflammation, cell survival and tumorigenesis. Both the NF-κB members and the NF-κB regulators play crucial roles in NF-κB regulation, and the maintenance of normal NF-κB signaling is critical in cancer prevention.

1.2.8 NF-κB in virus-mediated cancer

NF-κB signaling is also involved in tumors caused by viruses including HTLV-I, Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8), Epstein-Barr virus (EBV) and hepatitis B virus (HBV) (Sun and Xiao, 2003). As described before, HTLV-I causes ATL, and its coded oncoprotein Tax is crucial to HTLV-I-mediated NF-κB activation and tumorigenesis. Tax activates both canonical and non-canonical NF-κB signaling pathways in similar but not identical manners through interacting with IKKγ, the regulatory subunit of the IKK complex, resulting in the IKK activation (Matsuoka and Jeang, 2007; Sun and Yamaoka, 2005; Yoshida, 2010).

KSHV/HHV-8 is associated with different clinical variants of Kaposi's sarcoma, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Ganem, 2010; Moore

and Chang, 2003). In KSHV-infected PEL cells, both NF-κB pathways are constitutively activated by the viral protein vFLIP. Similarly to the HTLV-I oncoprotein Tax, vFLIP induces NF-κB activation through an interaction with IKKγ, although the role of β-TrCP in vFLIP mediated p100 processing remains to be elucidated (Ganem, 2010; Liu *et al.*, 2002; Matta and Chaudhary, 2004). Moreover, vFLIP-mediated IκB degradation requires IKKα but not IKKβ. Also, the suppression of p100 processing by knocking down vFLIP causes a significant reduction in the growth and proliferation of the malignant cells (Matta and Chaudhary, 2004).

EBV is a member of the herpesvirus family that is associated with human cancers including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma and gastric carcinoma (Cesarman and Mesri, 2007; Sun and Xiao, 2003). It efficiently immortalizes human B cells through a method largely mediated by the encoded latent membrane protein 1 (LMP1). LMP1 functions to mimic the constitutively activated members of the TNFR (the tumor necrosis factor receptor) superfamily, the stimuli for both canonical and non-canonical NF-κB pathways, resulting in the constitutive activation of NF-κB (Lam and Sugden, 2003).

HBV infection is responsible for acute and chronic hepatitis (Sun and Xiao, 2003). Unlike HTLV-I, in which Tax plays a major role in regulating cellular signaling, several HBV-encoded proteins are involved in NF-κB activation. These activators include the X transactivator protein (HBx), C-terminal-truncated middle-size surface proteins (MHBst) and the large HBV surface protein (LHBs). HBx activates NF-κB through indirectly activating IKK by targeting Ras and PKC signaling, or competing to interact with IκB so as to release the IκB-bound NF-κB dimers. However, MHBst and LHBs cooperate to form PreS2, the HBV activator proteins,

subsequently inducing the activation of transcription factors, including AP-1 and NF-κB (Hildt *et al.*, 1996; Hiscott *et al.*, 2001; Kekule *et al.*, 1993; Meyer *et al.*, 1992; Sun and Xiao, 2003).

Human papillomavirus (HPV) infection is also linked to NF-κB activation (Nair and Pillai, 2005). HPV causes various cancers including cervical cancer, lung cancer, penile cancer and anal cancer. Importantly, only a few of the numerous subtypes of HPV contribute to the risk of cancer development. It has been reported that NF-κB activation contributes to HPV-associated tumorigenesis and that the inhibition of NF-κB is an efficient method of cervical cancer treatment. Unlike HTLV-I Tax, the HPV oncoprotein E5 indirectly activates NF-κB by targeting EGFR. The role of NF-κB in HPV infection is at the center of a controversy because other HPV oncoproteins, E6 and E7, actually serve to inhibit NF-κB by inactivating p300 (Woodworth, 2002).

1.3 PDLIM2 AND ITS CELLULAR FUNCTIONS

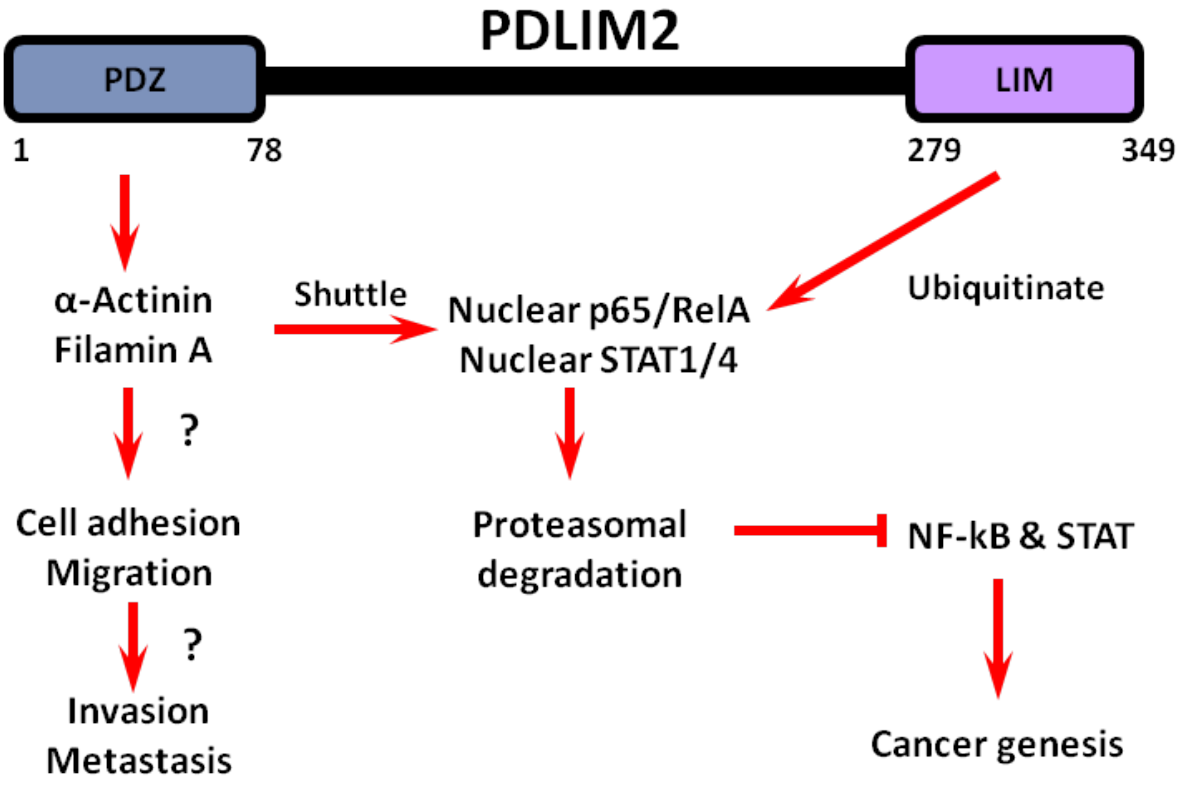

Figure 10. Schematic display of functional regions in PDLIM2.

PDLIM2 belongs to the actinin-associated LIM protein (ALP) subfamily. It was initially discovered in the cDNA library of rat eyes (Torrado *et al.*, 2004). Through a yeast two-hybrid screen, PDLIM2 was also identified as a STAT binding protein (Tanaka *et al.*, 2005). It has a PDZ domain in the N-terminus and a LIM domain in the C-terminus, which are responsible for most of the cellular functions of PDLIM2 including cytoskeletal interaction, IGF-related signaling transduction, cell adhesion and migration (Healy and O'Connor, 2009; Loughran *et al.*, 2005; Torrado *et al.*, 2004). Recently, PDLIM2 was characterized as playing a role in the

suppression of NF-κB signaling pathways and the STAT pathway by promoting the proteasomal degradation of p65 (RelA) and STAT1/STAT4 (Mankan *et al.*, 2009; Tanaka *et al.*, 2007; Tanaka *et al.*, 2005; Ungureanu and Silvennoinen, 2005). Additional evidence has shown that PDLIM2 contributes to oncogenesis by inhibiting the anchorage-independent growth of malignant cells, suggesting a potential function as a tumor suppressor (Loughran *et al.*, 2005; Qu *et al.*, 2010a; Qu *et al.*, 2010b; Yan *et al.*, 2009). Studies on HTLV-I HBZ have reported that HBZ suppresses p65-mediated NF-κB activation by activation of PDLIM2, linking PDLIM2 to HTLV-I (Zhao *et al.*, 2009). The cellular functions of PDLIM2, a novel defined protein with limited known features, are recapitulated and predicted in the following sections.

1.3.1 PDLIM2 and ALP protein subfamily

So far, nine proteins have been characterized as containing both the PDZ domain and one or more LIM domains (Te Velthuis *et al.*, 2007). Seven of them have their PDZ domains in the N-termini. Depending on the number of C-terminal LIM domains, they are classified into two groups, called the ALP and Enigma protein subfamily. The ALP protein subfamily, which includes Elfin (PDLIM1, CLP36, or CLIM), PDLIM2, ALP (PDLIM3) and RIL (PDLIM4), contains a single LIM domain, while Enigma protein subfamily members like ENH (PDLIM5), ZASP (PDLIM6, Oracle or Cypher) and Enigma (PDLIM7 or LMP-1) have three C-terminal LIM domains (Te Velthuis and Bagowski, 2007; Te Velthuis *et al.*, 2007; Zheng *et al.*, 2010). Evidence has shown that all six proteins other than Enigma can interact with α-actinin via their N-terminal PDZ domains (Elkins *et al.*, 2010). Generally, proteins containing both PDZ and LIM domains can interact with cytoskeletal proteins and play roles in development, cytoskeletal organization and oncogenesis (Te Velthuis and Bagowski, 2007). As reported, Enigma, ALP and

ZASP contribute to bone morphogenesis and the development of muscle and the heart, while PDLIM2 and RIL are associated with the regulation of cellular signaling transduction, cell adhesion, cell migration and carcinogenesis (Boumber *et al.*, 2007; Kang *et al.*, 2000; Kiess *et al.*, 1995; Pashmforoush *et al.*, 2001; Tanaka *et al.*, 2007; Tanaka *et al.*, 2005; van der Meer *et al.*, 2006; Zhou *et al.*, 2001). As opposed to all other members, PDLIM2 specifically plays roles in the proinflammatory response and the immune system.

The PDZ domain is a homology domain identified in DLG1, PSD95 (the postsynaptic density protein) and the epithelial tight junction protein ZO-1 (Fanning and Anderson, 1999; Te Velthuis *et al.*, 2007). It is a small protein domain with 80-90 amino acids and is responsible for protein-protein and protein-phospholipid interactions (Jelen *et al.*, 2003; Te Velthuis and Bagowski, 2007). A typical PDZ domain consists of six anti-parallel β strands and two helixes and recognizes up to seven amino acids of the target protein (Elkins *et al.*, 2010; Tonikian *et al.*, 2008). Moreover, recent studies on PDLIM2 and p65 suggest that the PDZ domain has additional functions in intra-nuclear trafficking (Tanaka *et al.*, 2007). Importantly, a lot of virus-encoded oncoproteins, including HTLV-I Tax and HPV-16 E6, contain the PDZ-domain binding motif (PBM). This motif is considered to contribute to the virus-mediated cell transformation and oncogenesis. Results have shown that the existence of mutations on PBM can affect viral infectivity and might be used to distinguish high-risk or low-risk oncoviruses (Aoyagi *et al.*, 2010; Feuer and Green, 2005).

The LIM domain is a cysteine-histidine-rich, double-tandemly repeated zinc-finger domain (Te Velthuis *et al.*, 2007). Similarly to the PDZ domain, the LIM domain provides a protein-protein interface and is associated with the biological functions of organ development

and oncogenesis. Aberrant expressions of LMO1 and LMO2 proteins, which contain only the LIM domain, are associated with human disease including T cell leukemia (Bach, 2000). Moreover, studies on PDLIM2 have shown that the LIM domain also serves as the ubiquitin E3 ligase to trigger the poly-ubiquitination and proteasomal degradation of nuclear p65 (Tanaka *et al.*, 2007).

1.3.2 Ubiquitin E3 ligase function of PDLIM2

It has been shown that PDLIM2 can be auto-ubiquitinated *in vitro* and that the C-terminal LIM domain of PDLIM2 is structurally similar to the RING finger domain (Capili *et al.*, 2001), which normally binds ubiquitination enzymes and substrates and functions as a ligase (Lorick *et al.*, 1999). In coincidence with these features, PDLIM2 plays roles in STAT and NF-κB signaling by targeting STAT1/STAT4 and p65 for poly-ubiquitination and proteasomal degradation, which is unique among all other PDZ-LIM-containing proteins. Poly-ubiquitination with subsequent proteasomal degradation is the main mechanism for a host cell to clean out useless proteins and shut down signal transduction. Three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), are required for protein ubiquitination. Of note, ubiquitin E3 ligases contribute to the specificity of the reaction as they selectively recruit the specific protein's substrate in the final step to transfer the poly-ubiquitin chain from the E2 enzyme to the target. After that, the poly-ubiquitinated target protein can be recognized and degraded by the proteasome (Pickart and Eddins, 2004; Xiao, 2007). An *in vitro* ubiquitination assay using a reconstitution system with recombined E1, E2 and PDLIM2 further confirms the ubiquitin E3 ligase function of PDLIM2 (Tanaka *et al.*, 2005). Correlated with the functional regulation of STAT1/STAT4 and p65, PDLIM2 substrates show elevated basal

expression and related signaling activation in PDLIM2-null cells and mice (Tanaka *et al.*, 2007; Tanaka *et al.*, 2005).

1.3.3 Other biological functions of PDLIM2

Severe defects are caused by deficiencies of other ALP and Enigma subfamily members like ALP, ZASP and RIL. ALP-null mice exhibit embryonic chamber dysmorphogenesis as well as cardiomyopathy in adult mice (Pashmforoush *et al.*, 2001); the ablation of ZASP in mice exhibits a phenotype of embryonic or perinatal lethality and is associated with disorganized and fragmented Z-lines in skeletal and cardiac muscle (Zhou *et al.*, 2001); a single genomic variation in the RIL gene promoter is associated with a low bone mineral density (BMD) in Japanese women (Omasu *et al.*, 2003). Distinct from the findings above, PDLIM2-deficient mice can be born healthy with normal numbers of lymphocytes, macrophages and dendritic cells (Tanaka *et al.*, 2005). Importantly, the ablation of PDLIM2 in cells leads to increased cytokine production and a susceptibility to LPS (Tanaka *et al.*, 2007). PDLIM2 is currently considered to play pivotal roles in the regulation of inflammation, the immune response and tumorigenesis when considering its contributions to the NF-κB and STAT pathways.

Although PDLIM2 is a newly defined protein with limited data, it has been shown that PDLIM2 interacts with both α-actinin and Filamin A, is regulated by IGF and is associated with cell adhesion and migration (Loughran *et al.*, 2005; Torrado *et al.*, 2004). The activation of the IGF-I receptor tyrosine kinase, which responds to IGF, promotes cell survival, migration and mitogenesis. The association between PDLIM2 and IGF-I is characterized by the utilization of IGF-IR-deficient cells. Studies show that IGF-I induces PDLIM2 expression, and breast cancer

MCF-7 cells with high PDLIM2 expression present elevated cell adhesion to ECM proteins but reduced colony formation ability *in vitro*. Moreover, based on evidence from studies on point mutations within the PDZ and LIM domains, both domains have been shown to be required to reverse the suppression of colony formation by PDLIM2, although a mutation in the PDZ domain itself is sufficient to eliminate promoted adhesion (Loughran *et al.*, 2005). Furthermore, knocking down PDLIM2 mRNA by siRNA shows an abrogation of both cell adhesion and migration in MCF10A and MCF-7 cells (Loughran *et al.,* 2005). All of this evidence indicates a crucial role for PDLIM2 in cell migration capacity as well as in tumor prevention (Loughran *et al.*, 2005; Te Velthuis and Bagowski, 2007).

It should be noted that most current studies on PDLIM2 are restricted to over-expression conditions, while PDLIM2 is predominantly expressed in the nucleus under physiological conditions (Tanaka *et al.*, 2005). A recent study suggested that the expression level and subcellular distribution of PDLIM2 are associated with the differentiation from monocytes to macrophages (Healy and O'Connor, 2009). Furthermore, the cytoplasmic sequestration of PDLIM2 has been shown to be a positive event in the regulation of NF-κB, which leads to decreased cell adhesion and promotes NF-κB activity (Healy and O'Connor, 2009). However, the model cannot exclude the possibility that the outcome actually indirectly results from the regulation of STAT and NF-κB by PDLIM2. Interestingly, as described above, the re-introduction of PDLIM2 inhibits the colony formation of breast cancer cells. Additionally, considering that PDLIM2 negatively regulates NF-κB and that NF-κB plays a pivotal role in tumorigenesis, it will be fascinating to investigate whether PDLIM2 has a potential tumor suppression function.

1.3.4 Potential tumor suppression function of PDLIM2

PDLIM2 is ubiquitously expressed in most tissues, with the highest expression in the lung, modest expression in motile epithelial cells, the thymus, the kidney and the spleen and low expression in the brain, the heart and the muscle (Loughran *et al.*, 2005; Tanaka *et al.*, 2005; Torrado *et al.*, 2004). However, the expression of PDLIM2 is suppressed in multiple carcinomas generated from T cells, the breast, the lung and the colon (Loughran *et al.*, 2005; Qu *et al.*, 2010a; Qu *et al.*, 2010b; Yan *et al.*, 2009). Also, the re-introduction of PDLIM2 largely suppresses the colony formation of those cells *in vitro* and tumor growth in SCID mice, suggesting PDLIM2 repression as a general event in cancer progression and as a novel potential biomarker of cancer. Meanwhile, in accordance with its function as a terminator of NF-κB, it is plausible that PDLIM2 serves as a tumor suppressor by inhibiting NF-κB (Healy and O'Connor, 2009; Tanaka *et al.*, 2007).

Although the mechanism by which the *PDLIM2* gene is largely repressed in those cancer cells remains under investigation, one clue is that the genomic locus of PDLIM2, 8p21, frequently undergoes allelic loss in ovarian and prostate cancers (Brown *et al.*, 1999; Swalwell *et al.*, 2002). Another possibility involves the epigenetic suppression of PDLIM2, which requires the hyper-methylation of the *PDLIM2* promoter (Qu *et al.*, 2010a; Qu *et al.*, 2010b). It has been reported that the tumor suppressor RIL (PDLIM4), another ALP subfamily member, is repressed by hyper-methylation of DNA at its promoter region in ~70% (55/79) of cancer cell lines of tested prostate tumors (Vanaja *et al.*, 2006). Because it is in the same protein subfamily, it is plausible that PDLIM2 may also be a potential novel tumor suppressor that is repressed by a similar epigenetic mechanism in cancers.

1.4 CONCLUDING REMARKS

HTLV-I infection caused adult T cell leukemia is an NF-κB related disease. Although the detail mechanisms of ATL progression are under investigation, it is accepted that HTLV-I oncoprotein Tax plays a crucial role in HTLV-I viral infection, replication, cell transformation and the early stage of ATL progression. The oncogenic functions of Tax have been extensively studied, including degenerating intracellular signal transduction, immortalizing primary T lymphocytes, inducing aneuploidy of ATL cells, inhibiting tumor suppressor genes and overcoming cell-cycle. NF-κB is the chief target of Tax and well-known for its function in the inflammatory response, cell survival, cell growth and oncogenesis. Inhibition of NF-κB has been used for drug discoveries against lots of diseases including ATL.

PDLIM2 responses to IGF-I, serves as a novel E3 ligase of STATs and p65, and contributes to cell adhesion and cell migration. It serves as the terminator of both STAT and NF-κB pathways, and plays a crucial role in immune response. Recent studies on HTLV-I HBZ have linked the PDLIM2 to HTLV-I. In this thesis, we will reconcile the association between HTLV-I Tax and PDLIM2 based on their distinct functions on NF-κB signaling.

We found that HTLV-I Tax is directly and negatively regulated by PDLIM2. PDLIM2 not only suppressed Tax-mediated NF-κB signal transduction and Tax-mediated tumorigenesis, but may play a role in determination of the outcome of HTLV-I infection (Chapter 2). Later, the molecular determinants within both PDLIM2 and Tax were investigated by taking advantages of the deletion or substitution mutants of PDLIM2 and Tax. A putative α-helix region within PDLIM2 was defined to be responsible for the interaction between PDLIM2 and Tax, while the C-terminal LIM domain of PDLIM2 was required for the E3 ligase function of PDLIM2

(Chapter 3). Furthermore, we reported that the HTLV-I-mediated repression of PDLIM2 was associated with HTLV-I-mediated tumorigenesis and involved DNA methylation (Chapter 4). Our results characterized PDLIM2 as the first intrinsic negative regulator of HTLV-I Tax to defend HTLV-I infection and the counterbalance between PDLIM2 and Tax may determine the outcome of HTLV-I infection, providing insights into HTLV-I leukemogenicity and the long-latency of ATL.

2.0 PDLIM2 TARGETS TAX FOR PROTEASOMAL DEGRADATION AND SUPPRESSES HTLV-I/TAX-MEDIATED TUMORIGENESIS

Portions of research described in this section were published in *Blood (Blood. 2009,* 113(18): 4370-80) with authors Pengrong Yan, Jing Fu, Zhaoxia Qu, Shirong Li, Takashi Tanaka, Michael J Grusby and Gutian Xiao.

2.1 SUMMARY

The mechanisms by which the human T cell leukemia virus type I (HTLV-I) Tax oncoprotein deregulates cellular signaling for oncogenesis have been extensively studied, but how Tax itself is regulated remains largely unknown. Here we reported that Tax was negatively regulated by PDLIM2, which promoted Tax poly-ubiquitination and proteasomal degradation. In agreement with that, PDLIM2 suppressed Tax-mediated signaling activation, cell transformation and oncogenesis both *in vitro* and in animal. Additionally, re-introduction of PDLIM2 reversed the tumorigenicity of the HTLV-I-transformed T cells. Our studies characterize PDLIM2 as the first intrinsic negative regulator of HTLV-I Tax, which may contribute to the outcome of HTLV-I infection. These studies also suggest a potential therapeutic strategy for cancers and other diseases associated with HTLV-I infection and/or PDLIM2 deregulation.

2.2 INTRODUCTION

As described in the General Introduction, HTLV-I is a retrovirus which causes ATL (Yoshida, 2010). Its encoded Tax protein is oncogenic and crucial for HTLV-I viral replication, transmission and HTLV-I-mediated pathogenesis (Yoshida, 2010). HTLV-I without Tax loses its original transformative ability, indicating that Tax is indispensable (Yamaoka *et al*., 1992). Tax not only transforms and immortalizes cells *in vitro*, it also induces tumor formation in transgenic mice as well (Grassmann *et al*., 1992; Pozzatti *et al*., 1990; Tanaka *et al*., 1990; Grossman *et al*., 1995). Tax exerts its oncogenic functions by modulating lots of cellular transcription factors including NF-κB and CREB (Grassman *et al*., 2005; Sun and Yamaoka, 2005; Yoshida, 2010). Importantly, NF-κB is considered to be an essential mediator of the Tax regulated cellular program, and is constitutively activated in HTLV-I-transformed T cells and primary ATL cells. The NF-κB activation by Tax serves as a pertinent step in HTLV-I-mediated T cell transformation (Sun and Yamaoka, 2005).

Tax contributes to the aberrant NF-κB activation at multiple levels (Matsuoka and Jeang, 2007; Sun and Yamaoka, 2005). In the cytosol, it interacts with IKKγ for IKK activation, leading to the degradation of IκB and processing of p100 to p52, which in turn results in the nuclear translocation of NF-κB dimers (Xiao *et al*., 2006). In the nucleus, full NF-κB activation requires further interactions between Tax and other transcriptional components including p65 and CBP/p300 (Xiao *et al*., 2006). Although the mechanisms by which Tax contributes to HTLV-I

transcription and deregulates cellular signaling pathways have been extensively studied, the intrinsic negative regulator of Tax itself is under scrutiny.

The following results characterize PDLIM2, a novel ubiquitin E3 ligase of STATs and p65, as a repressor of HTLV-I Tax (Tanaka *et al.*, 2007; Tanaka *et al.*, 2005). We show that PDLIM2 inhibits the expression level of Tax by promoting the poly-ubiquitination and proteasomal degradation of Tax, so to suppress Tax-mediated NF-κB activation, LTR transcription and oncogenesis. Moreover, exogenous expression of PDLIM2 in HTLV-I-transformed T cells inhibits the malignancy of the cells. Our studies define PDLIM2 as a novel intrinsic negative regulator of HTLV-I Tax, which may contribute to the outcome of HTLV-I infection.

2.3 MATERIALS AND METHODS

Expression vectors and reagents

Expression vectors encoding Tax, PDLIM2 and its LIM domain deletion mutant have been described before (Qu *et al.*, 2004; Tanaka *et al.*, 2007). Ubiquitin construct was gifts of Dr. James Z. Chen. The Tax and PDLIM2 cDNAs were also sub-cloned into retroviral vectors pCLXSN and/or pTRIP by routine cloning strategies as described (Qing *et al.*, 2005a). The HA monoclonal antibody (12CA5) and HRP-conjugated HA monoclonal antibody (3F10) were from Roche Molecular Biochemicals. The ubiquitin, Sp1, lamin B, Hsp90, and β-actin antibodies as well as the pre-immune IgG were from Santa Cruz Biotechnology. The proteasome inhibitor MG132 and protein synthesis inhibitor cycloheximide (CHX) were from Biomol. The

endocytosis inhibitor monodansyl cadaverine (MDC), caspase inhibitor N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (z-VAD-FMK) and autophagy inhibitor 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) were from Sigma. The Tax and MYC antibodies were described previously (Qu *et al.*, 2004).

Cell culture and transfection

HEK293 cells, Rat-1 fibroblasts and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 2 mM L-glutamine (Invitrogen). Human T lymphocyte Jurkat, and HTLV-I-transformed T cell lines C8166 and MT-4 were maintained in suspension in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Yan *et al.*, 2007). 293 and Jurkat cells were transfected with DEAE-Dextran (Sigma), MEF cells with Lipofectamine 2000 (Invitrogen), and HTLV-I-transformed T cells with Transfast reagent (Promega) (Qing and Xiao, 2005; Xiao and Sun, 2000).

Retroviral transduction and generation of stable transfectant

Rat-1 cells and HTLV-I-transformed T cells were infected with virus expressing Tax or PDLIM2, respectively. The Rat-1 cells stably expressing Tax were also re-infected with virus expressing PDLIM2 for simultaneously expressing both Tax and PDLIM2. The viruses expressing GFP were used as a control. The stable transfectants were obtained by selection with G418 and/or blasticidin selections as described previously (Qing *et al.*, 2007).

Immunoblotting (IB) and immunoprecipitation (IP) assays

Whole-cell extracts were prepared by lysing cells in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 0.25% (wt/vol) Na-deoxycholate, 1% (vol/vol) NP-40, 1 mM DTT). All the lysis buffers were supplemented with 1 mM PMSF and a protease inhibitor cocktail (Roche Molecular Biochemicals). The cells extracts were used for IP and/or IB assays as described before (Qing *et al.*, 2007).

In vivo ubiquitin conjugation assay

Protein extracts were prepared from 293 cells transfected with Tax together with HA-tagged ubiquitin in the presence or absence of PDLIM2, immediately followed by IP using anti-Tax. The ubiquitin-conjugated Tax pulled down by IP was detected by IB using anti-HA-HRP (Xiao *et al.*, 2001b).

Protein stability assay

Cells were treated with 10 μM CHX, followed by chasing of the indicated time period in the presence or absence of MG132, and IB to detect the indicated proteins (Qing *et al.*, 2005b).

Cell cycle analysis

C8166 and MT4 cells were washed twice with 1X PBS, fixed by treatment with ice-cold 70% ethanol for 30 min on ice and stored at 4° C prior to analysis. For staining, cells were incubated in PBS containing 1 mg/ml RNaseA, 40 μg/ml propidium iodide (Sigma-Aldrich) for 30 min in the dark at 37° C and then analyzed by flow cytometry. For each sample, more than 3 x 10^4 cells were counted, and the cells with a lower DNA content than those of the G_0/G_1 phase

were referred to dead cells. DNA histograms were analyzed using ModFit (Verity Software House, Topsham, ME) software.

RT-PCR analysis

Total RNA was prepared with TRIZOL reagent and cDNA was generated with SuperScript II reverse transcriptase (Invitrogen), followed by PCR assays as described before (Qing *et al.*, 2005b, 2007). Primer pairs for RT-PCR were as follows: human PDLIM2, forward 5'- TCTCACCACCACCCTCTAGC, human PDLIM2, reverse 5'-CTTCAGGTTCAGCCCACAGT; mouse PDLIM2, forward 5'-GACAGCCAGTCTTCCCAGAG, mouse PDLIM2, reverse 5'-TCTCACAGGTGTGGAGCTTG; Tax, forward, 5'-CACCTGTCCAGAGCATCAGA; Tax reverse, 5'-CGCTTGTAGGGAACATTGGT; GAPDH, forward 5'-CACAGTCCATGCCATCACTG, GAPDH, reverse 5'-CTTACTCCTTGGAGGCCATG;

Luciferase gene reporter assays

Jurkat, 293 and MEF cells were transfected with luciferase reporter and Tax in the presence of increasing amounts of PDLIM2. For MT-4 cells, the luciferase reporter together with increasing amounts of PDLIM2 was transfected. At 40 hrs post-transfection, luciferase activity was measured as we described before (Xiao *et al.*, 2000).

Colony formation assays

Soft agar assays were performed as previously described (Qing *et al.*, 2007). Briefly, 6-well plates were coated with an initial underlay of 1% SeaPlaque low melting agarose in culture

medium. Cell suspension in culture medium containing 0.6% SeaPlaque low melting agarose was then added to the coated plates. Colony growth was scored after 21 days of cell incubation at the normal condition. All the colony formation assays presented in this study were repeated in at least three independent experiments.

In vivo tumorigenicity assays

Five-week old female SCID mice (C.B-17^{TM}/IcrCrl-scidBR) (from Charles River Lab.) were challenged subcutaneously in hind-back with Rat-1 stable cell lines, or subcutaneously in the post-auricular region with C8166 or MT-4 stably expressing PDLIM2 or an empty vector. The recipient mice were monitored, sacrificed and dissected for tumor evaluation at the indicated post-injection days.

Statistical significance

Paired student's t-test for independent samples was used to assess statistical significance.

2.4 RESULTS

2.4.1 PDLIM2 suppresses Tax-mediated NF-κB activation and HTLV-I viral transcription.

2.4.1.1 PDLIM2 suppresses Tax-mediated NF-κB activation.

Tax-induced NF-κB activation is crucial for HTLV-I-mediated pathogenesis, particularly the cell transformation and tumorigenesis, and PDLIM2 could function as a terminator of NF-κB

by triggering the degradation of p65 (Sun and Yamaoka, 2005; Tanaka *et al.*, 2007). Therefore, we examined the effect of PDLIM2 on Tax-induced NF-κB in luciferase gene reporter assay to test whether PDLIM2 could also inhibit Tax-mediated NF-κB activation. As expected, the expression of Tax efficiently activated NF-κB transcription activity in both 293 and Jurkat cells (Figure 11A, 11B). PDLIM2 co-expression induced a dose-dependent suppression on the Tax-mediated activation of NF-κB in 293 and Jurkat T cells. Importantly, similar results were obtained in MT-4 cells, an HTLV-I-transformed T cell line which constitutively expresses endogenous Tax protein (Figure 11C).

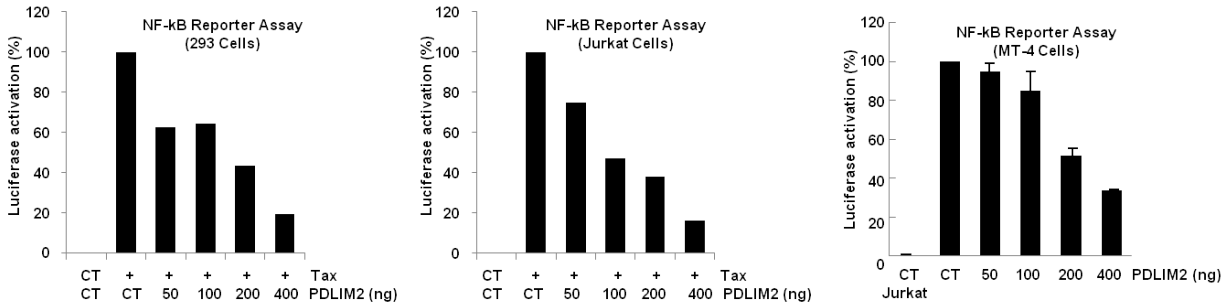

Figure 11. PDLIM2 inhibits Tax-dependent NF-κB activation.

(A, B, C) The indicated cells were transfected with Tax and κB driven luciferase reporter in the presence of increasing amounts of PDLIM2, followed by the measurement of luciferase activity. To eliminate the effect of CMV promoter on NF-κB activations, the Tax and PDLIM2 used here are both under RSV promoter.

2.4.1.2 PDLIM2 suppresses Tax-mediated HTLV-I-LTR viral transcription.

Whereas Tax-mediated NF-κB activation is largely responsible for HTLV-I-mediated oncogenesis, the transactivation of HTLV-I transcription by Tax is important for HTLV-I viral gene expression and viral replication (Matsuoka and Jeang, 2007). We utilized a luciferase gene reporter driven by the HTLV-I LTR. Similar to the former result, PDLIM2 suppressed Tax-

mediated HTLV-I viral transcription in a dose-dependent manner (Figure 12). Taken them together, the results suggest that PDLIM2 negatively regulates Tax-induced NF-κB and HTLV-I-LTR activation. Importantly, because the transcriptional activation by Tax is independent of NF-κB, there might be a direct connection between PDLIM2 and Tax.

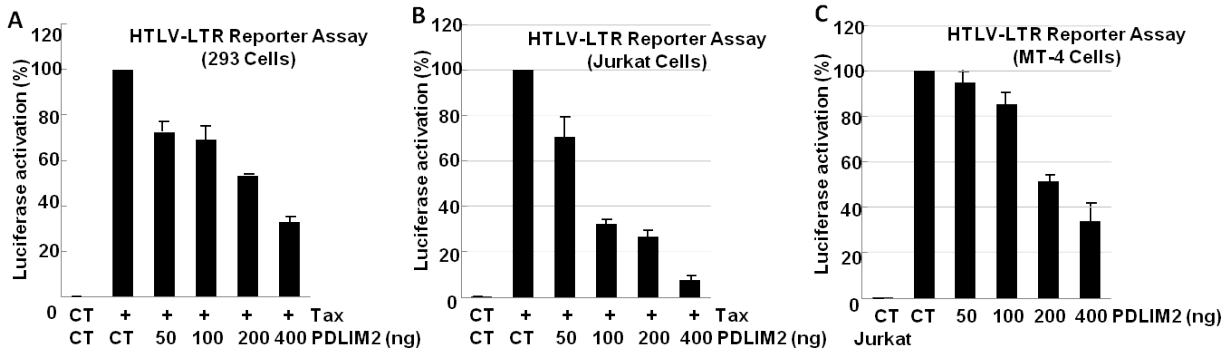

Figure 12. PDLIM2 inhibits Tax-dependent HTLV-I-LTR viral transcription.

(A, B, C) The indicated cells were transfected with Tax and HTLV-I-LTR driven luciferase reporter in the presence of increasing amounts of PDLIM2, followed by the measurement of luciferase activity.

2.4.2 PDLIM2 inhibits HTLV-I Tax-mediated tumorigenesis.

2.4.2.1 Co-expression of PDLIM2 inhibits Tax-mediated anchorage-independent colony formation and tumor growth in SCID mice.

As described before, the tumorigenicity of HTLV-I is largely mediated by HTLV-I-encoded Tax protein at multiple levels. Considering that Tax mainly functions through NF-κB and HTLV-I-induced ATL is an NF-κB-related disease, it is necessary to examine whether PDLIM2-induced NF-κB suppression also affects Tax-mediated oncogenesis. Rat-1 cell is a good model cell to address the issue because Rat-1 cell has been demonstrated to acquire the

ability to form foci in soft agar when infected with Tax (Tanaka *et al.*, 1990). In agreement with the negative role of PDLIM2 in Tax-mediated NF-κB activation and HTLV-I-LTR viral transcription, co-expression of PDLIM2 sufficiently inhibited the anchorage-independent growth of Tax-expressing Rat-1 cells, indicating that PDLIM2 suppresses the transformative ability of Tax *in vitro* (Figure 13A).

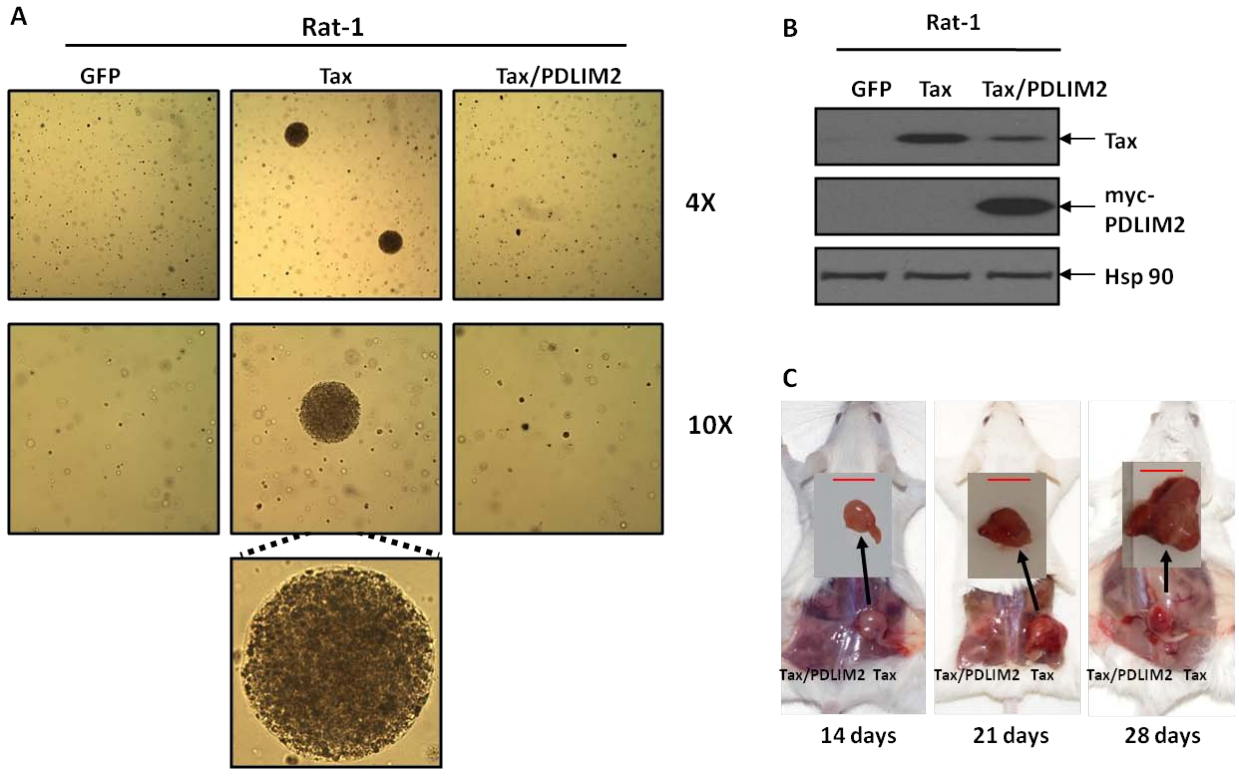

Figure 13. PDLIM2 suppresses Tax-mediated cell transformation and tumor growth in SCID mice.

(A) Rat-1 cells stably expressing GFP, Tax and Tax/PDLIM2 were plated in soft agar for colony formation assay. Pictures shown were taken at day 14 after plating. (B) Expression levels of Tax, exogenous PDLIM2 in Rat-1 stable cells used in panel A were examined by IB using Tax and myc antibodies, respectively. Hsp90 was used as the internal loading control. (C) Rat-I cells indicated in panel A were subcutaneously inoculated into the right and left hind-back of the same SCID mouse, respectively. After inoculation for 14, 21, and 28 days, mice were sacrificed and pictures were taken, respectively.

To further confirm the result in animals, the Rat-1 cells were subcutaneously injected into the SCID mice. As expected, only the mice injected with the cells expressing tax (Figure 13B), but not the control vector (not shown), developed xenografts. As such, Tax driven tumorigenesis was almost completely blocked by PDLIM2 co-expression. These studies demonstrated that PDLIM2 co-expression is sufficient to prevent tumorigenicity of Tax both *in vitro* and in SCID mice.

2.4.2.2 PDLIM2 inhibits tumor formation of HTLV-I-transformed T cells in SCID mice.

Table 1. PDLIM2 prevents tumor formation of HTLV-I-transformed T cells in SCID mice.

Cell line	Clone No.	No. of mice with tumor /No. of mice injected	Average size of tumors $(mm)^3$
C8166	Vector	2/3	73
	PDLIM2 #8	0/4	0
	PDLIM2#18	0/4	0
MT-4	Vector	3/4	93
	PDLIM2 #20	0/4	0

We also introduced exogenous PDLIM2 into HTLV-I-transformed T cells including C8166 and MT4 through lentiviral infection. Both of the cell lines formed tumors after being subcutaneously injected into the SCID mice (although not all). Tumor generation was however blocked when cells were inoculated onto PDLIM2 expressing cells, indicating that PDLIM2 suppresses HTLV-I-mediated tumorigenicity (Table 1). Importantly, such inhibition did not seem to result from changes in cell proliferation and apoptosis since cell-cycle arrest assays gave comparable results between cells with and without PDLIM2 expression (Figure 14).

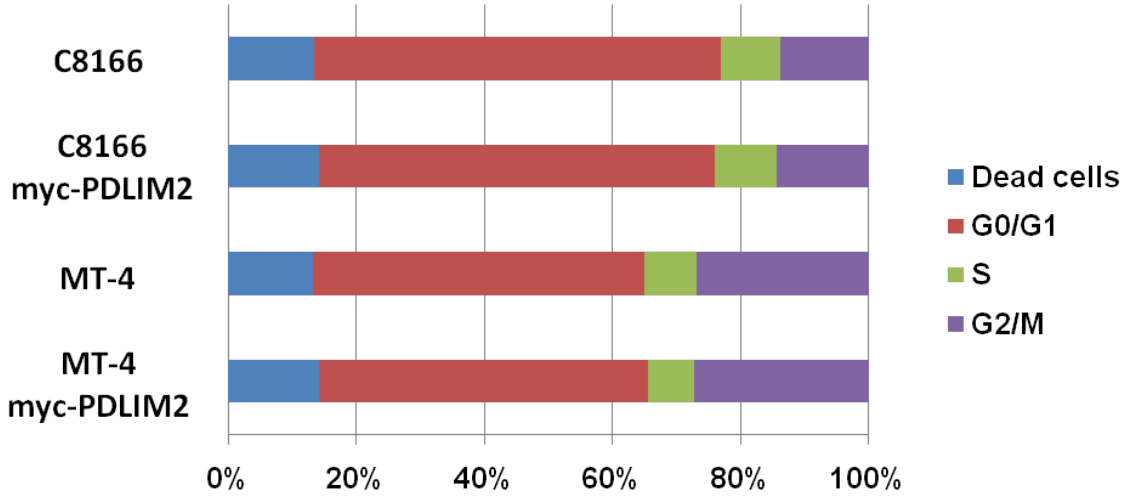

Figure 14. Exogenous introduction of PDLIM2 does not induce cell-cycle arrest in HTLV-I-transformed T cells.

The indicated HTLV-I-transformed T cell lines stably expressing PDLIM2 or the empty vector were cultured at the normal condition, stained with propidium iodide (PI) and subjected to FACS analysis.

2.4.3 PDLIM2 promotes the poly-ubiquitination and proteasomal degradation of Tax.

2.4.3.1 PDLIM2 regulates Tax expression in a dose-dependent manner.

Since PDLIM2 inhibits Tax-mediated HTLV-I-LTR transcription, an NF-κB independent event, our data suggests that PDLIM2 and Tax may share more than a signaling pathway and actually physically interact with one another. To define this association, we utilized different dosages of PDLIM2 and co-expressed it with Tax. As shown in Figure 15A, co-expression of PDLIM2 diminished the total Tax protein levels in a dose-dependent manner. This effect seemed to be directly caused by PDLIM2, since the exogenously expressed GFP and endogenous Hsp90 remained unaffected (Figure 15A). Furthermore, an examination of Tax mRNA levels suggested that PDLIM2 reduces Tax protein expression by serving at the post-transcriptional level because

there were little changes in Tax mRNA levels following the expression of PDLIM2 in 293 and MEF cells (Figure 15B).

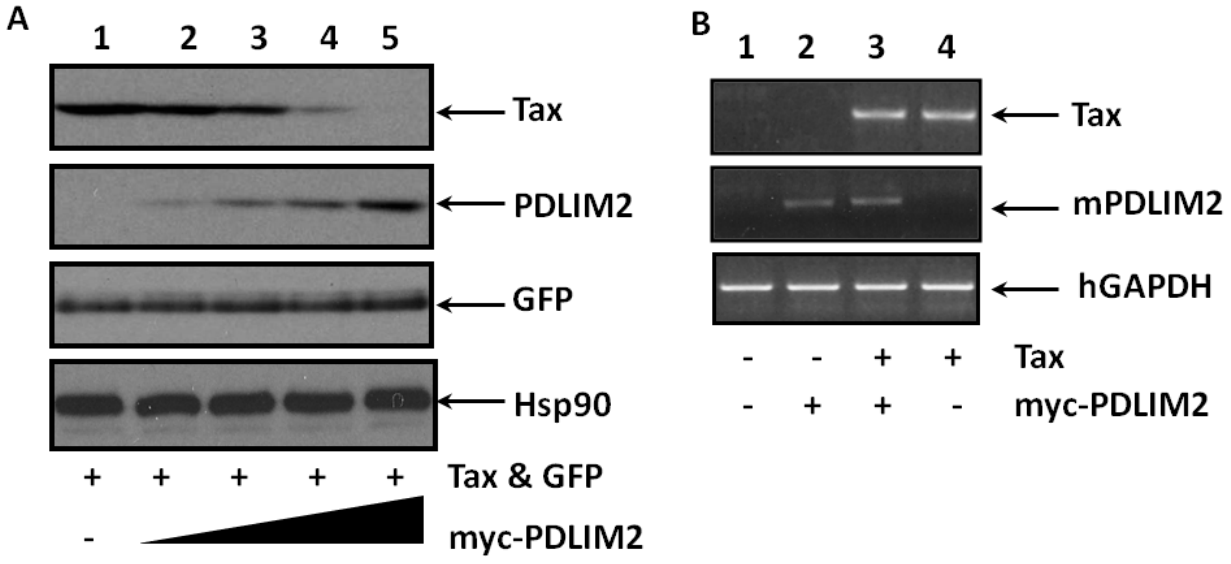

Figure 15. PDLIM2 represses Tax protein in a dose-dependent manner.

(A) 293 cells were transfected with Tax and GFP in the presence of increasing amounts of PDLIM2, following by IB to detect expression levels of Tax, myc-PDLIM2, GFP and Hsp90. Hsp90 was used as the internal loading control and GFP would indicate the transfection efficiency. (B) 293 cells were transfected with empty vector, Tax, Tax/PDLIM2 and PDLIM2 alone, following by RNA extraction and reverse transcription. Semi-quantification PCR for Tax, PDLIM2 and GAPDH were performed with four different amplification cycles.

2.4.3.2 Inducible expression of PDLIM2 results in reduced protein levels of Tax.

Transient transfection frequently increases experimental variability and gives artificial results. To overcome this issue and validate the former data, we generated PDLIM2-inducible cells in Tax expressing 293 cells, in which the expression of exogenous PDLIM2 was induced by doxycycline (Dox) treatment. As shown in figure 16A, following Dox treatment, PDLIM2 protein levels gradually accumulated, whereas Tax protein was almost completely degraded to

undetectable levels after 24hrs. We therefore proceeded to narrow the inducible time points. The most efficient suppressive period appeared between 6-12 hrs from Dox treatment (Figure 16B). In summary, PDLIM2 triggers Tax degradation and PDLIM2 protein levels are critical in determination of Tax protein levels.

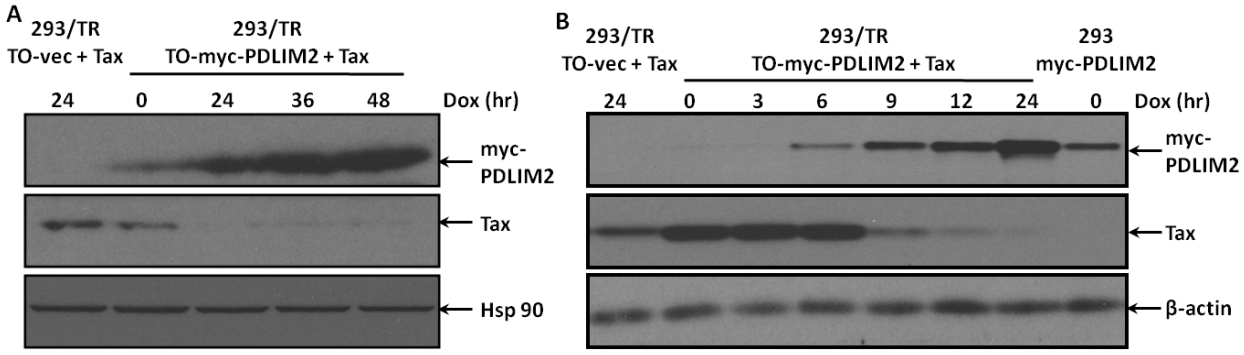

Figure 16. Inducible PDLIM2 suppresses Tax expression.

(A, B) PDLIM2-inducible 293 cells were mock-treated or treated with Dox for the indicated times followed by direct IB analysis using antibodies against Tax, myc and Hsp90. The Hsp90 and β-actin expression were used as the internal loading control. TR, Tetracycline Repressor; TO, Tetracycline Operator.

2.4.3.3 PDLIM2 directly interacts with Tax.

Based on those findings, we thought to determine whether PDLIM2 could interact with Tax. It is important to note that the C-terminal PDZ domain binding motif (PBM) within HTLV-I Tax is responsible for the interactions between Tax and various other PDZ-domain-containing proteins. Thus, it is possible that PDLIM2 interacts with Tax via its N-terminal PDZ domain. We performed co-immunoprecipitation (Co-IP) assays to address the question. When PDLIM2 and Tax expressed together in 293 cells, they indeed formed a stable complex (Figure 17A, 17B).

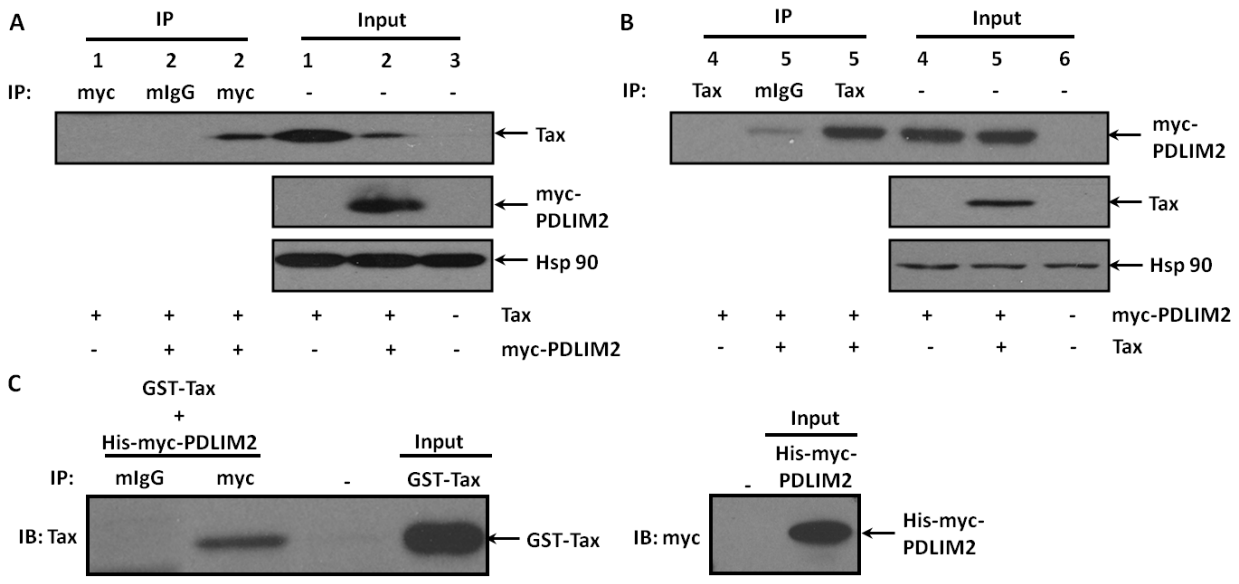

Figure 17. PDLIM2 directly interacts with Tax.

(A) 293 cells were transfected with Tax in the presence or absence of myc-PDLIM2, following by IP using myc antibody and IgG, and IB using Tax antibody. The expression of input Tax and myc-PDLIM2 were analyzed by direct IB. Hsp90 was used as the loading control. (B) Similar assay as panel A. (C) 2ug GST-Tax and 2ug His-myc-PDLIM2 proteins purified from *E. coli* were mixed together and incubated at 4^0C for 2 hrs, following by IP using myc antibody and IgG, and IB using Tax antibody.

To clarify whether the association was direct or indirect, we further examined the PDLIM2-Tax interaction through an *in vitro* Co-IP assay using recombinant PDLIM2 and Tax proteins purified from bacteria. As shown in Figure 17C, purified His-myc-PDLIM2 could not be pulled down by pre-immune IgG. However, recombinant Tax was easily detected using an anti-Tax antibody when incubated with His-myc-PDLIM2 and immunoprecipitated with an anti-Myc antibody. These results indicated that PDLIM2 directly interacts with Tax.

2.4.3.4 PDLIM2 triggers the proteasomal degradation of Tax.

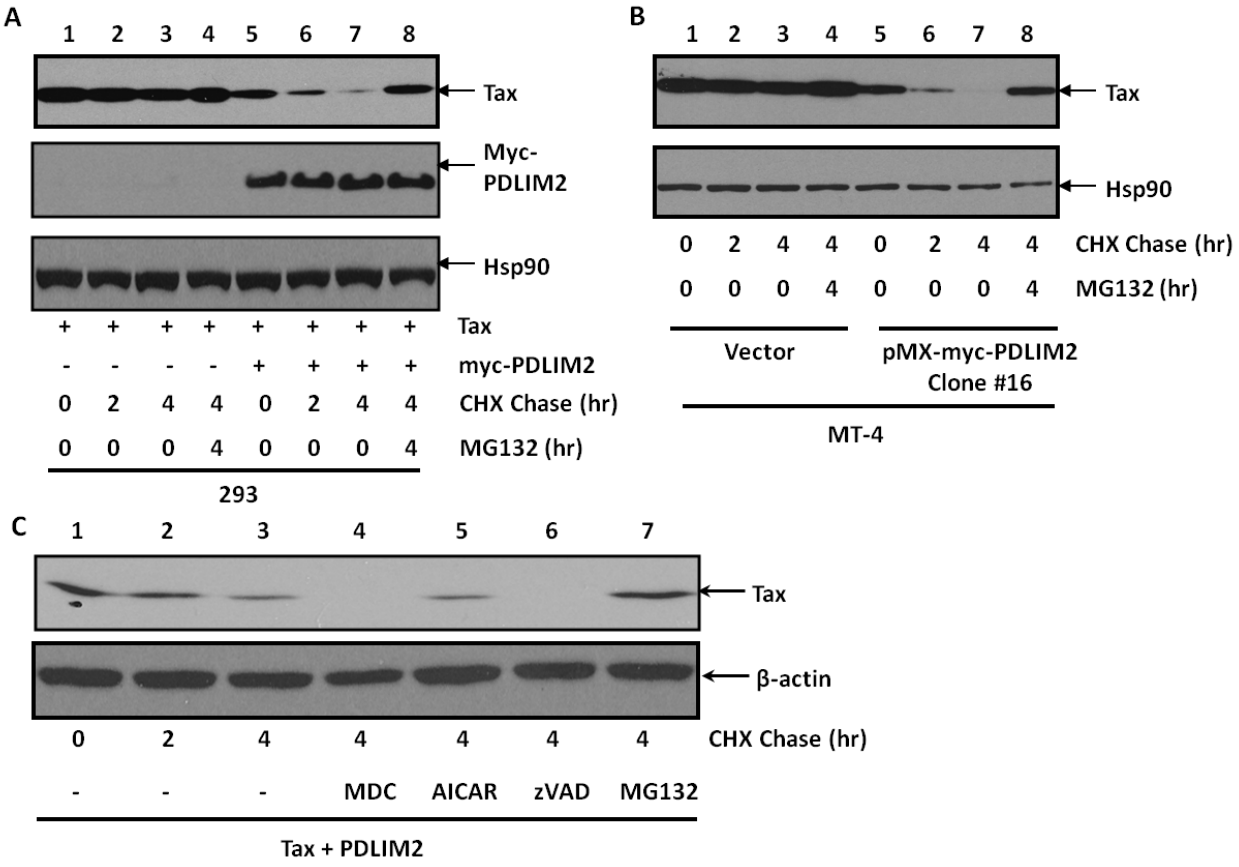

Figure 18. PDLIM2 promotes the proteasomal degradation of Tax.

(A) 293 cells transfected with Tax in the presence or absence of PDLIM2 were subjected to CHX-chase assay at the indicated time. In lane 4 and 8, the cells were chased in the presence of 10uM MG132, the proteasome inhibitor. (B) MT-4 cells stably expressing PDLIM2 or an empty vector were CHX chased as described before. (C) 293 cells transfected with Tax in the presence of PDLIM2 were subjected to CHX-assay at the indicated time. In lane 4-7, the cells were chased in the presence of MDC, AICAR, zVAD-FMK and MG132, respectively.

Given the low level of Tax protein when co-expressing PDLIM2, we though to determine whether PDLIM2 promoted the degradation of Tax. To confirm this hypothesis, the turnover of Tax protein was measured by performing CHX-chase assay in cells over-expressing PDLIM2. The results showed that PDLIM2 promoted Tax turnover in 293 cells as well as the HTLV-I-

transformed T cells (Figure 18A, 18B). Additionally, such elevated turnover could be suppressed by the treatment of MG132, the inhibitor of proteasome. It suggested that PDLIM2 might promote the proteasomal degradation of Tax.

We also ruled out the involvement of other mechanisms of protein degradation including endocytosis, autophagy and caspase-mediated protein degradation by using the specific chemical inhibitors. Following blocking cell endocytosis via treatment of monodansyl cadaverine (MDC), inhibiting autophagy by using 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) and turning off caspase-mediated protein cleavage by using carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-FMK), we measured Tax turnover rates. As shown in Figure 18C, none of these inhibitors, save MG132, could rescue Tax protein level after 4hrs CHX-chase. Therefore, our data indicates that PDLIM2 triggers the proteasomal degradation of Tax.

2.4.3.5 PDLIM2 promotes poly-ubiquitination of Tax.

Because protein ubiquitination is the major mechanism leading to protein proteasomal degradation, the above findings led us to investigate the mechanistic role of PDLIM2. Indeed, reports confirmed that PDLIM2 is a novel ubiquitin E3 ligase, whose enzymatic activity depends on its C-terminal LIM domain, a motif homologous to the RING finger domain (Tanaka *et al.*, 2007; Tanaka *et al.*, 2005).

To test whether PDLIM2 could promote the poly-ubiquitination of Tax or even directly function as an ubiquitin E3 ligase, we performed *in vivo* ubiquitination assays using 293 cells transfected with Tax and PDLIM2 plus wildtype (WT) exogenous ubiquitin. In agreement with

the reported data, Tax itself could be poly-ubiquitinated (Figure 19, lane 2; Nasr *et al*, 2006). When PDLIM2 was co-expressed, a significantly greater intensity of poly-ubiquitination signal was detected (Figure 19, lane 4 vs lane 2). Furthermore, using a similar assay, inhibition of proteasome by MG132 stabilized the poly-ubiquitinated Tax, indicating that proteasomal degradation of Tax involves PDLIM2 promoted Tax poly-ubiquitination (Figure 19, lane 6 vs lane 2, lane 8 vs lane 4).

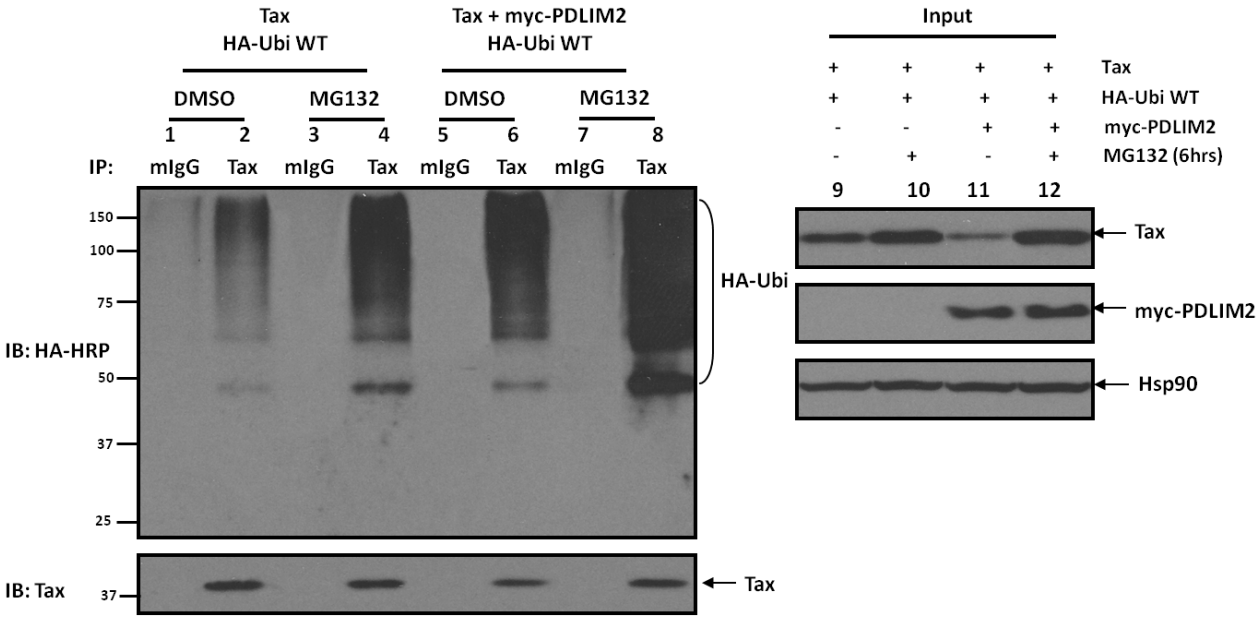

Figure 19. PDLIM2 promotes poly-ubiquitination of Tax.

293 cells transfected with HA-Ubiquitin (WT) and Tax in the presence or absence of PDLIM2 were subjected to IP by using mIgG and Tax antibodies, following by IB detection with HA-HRP antibody. In lane 3, 4, 7 and 8, the cells were treated with 10uM MG132 for 3hrs before performing IP. The pulling down efficiency and input expression of Tax were evaluated as indicated. Hsp90 was used as the loading control for the inputs.

2.5 DISCUSSION

Extensive studies have been performed on how Tax usurps cellular regulatory mechanisms to facilitate HTLV-I viral replication and to initiate malignant transformation leading to the development of ATL. However, how the Tax protein is regulated by cellular mechanisms has been rarely studied. Due to lack of this knowledge, the prognosis for this acute and fatal disease is still under investigation. Here we show that Tax is negatively regulated by PDLIM2.

PDLIM2 is a newly identified ubiquitin E3 ligase that could specifically poly-ubiquitinate the nuclear p65 and STAT proteins for proteasomal degradation (Tanaka *et al.*, 2007; Tanaka *et al.*, 2005). Similarly, our data indicates that PDLIM2 physically and directly interacts with HTLV-I Tax, triggers its proteasomal degradation by promoting its poly-ubiquitination. Considering the importance of Tax in HTLV-I viral transcription and tumorigenesis, PDLIM2-promoted proteasomal degradation of Tax leads to a turning down or shutoff of Tax-mediated signaling. Additionally, we found that PDLIM2 co-expression is sufficient to prevent *in vitro* cell transformation and *in vivo* oncogenesis induced by Tax, and the PDLIM2 re-introduction in HTLV-I-transformed T cells was able to reverse their tumor formation ability in animal. This evidence indicates that PDLIM2 might be the first cellular protein that binds to Tax but functions negatively.

Obviously, Tax degradation plays a predominant and direct role in PDLIM2 mediated suppression of Tax oncogenicity. However, given that PDLIM2 itself serves as a terminator of NF-κB signaling and also function in the downstream of Tax, our studies could not exclude the possibility that such suppressed tumorigenicity might result from the inhibition of NF-κB

through p65 degradation by PDLIM2 (Tanaka *et al.*, 2007). The relationship between Tax, PDLIM2 and NF-κB, especially the p65 mediated NF-κB activation, will be further elucidated in Chapter 5.

Our later studies found that PDLIM2 was largely repressed in HTLV-I-transformed T cells (Figure 30, Figure 31). Whereas HTLV-I repressed PDLIM2 via a yet-to-be elucidated mechanism, PDLIM2 targets HTLV-I Tax protein for degradation. Since the exogenous expression of PDLIM2 in HTLV-I-transformed T cells inhibits or reverses the malignancy, the counterbalance between Tax and host PDLIM2 may determine the outcome of HTLV-I-induced tumorigenesis and contributes to the long latency of HTLV-I-caused ATL. Our studies therefore suggest a novel therapeutic strategy for cancer and other diseases associated with HTLV-I infection and/or PDLIM2 deregulation. More discussions will be presented in Overall Discussion.

3.0 BOTH THE LIM DOMAIN AND THE MOTIF 236-254 ARE THE MOLECULAR DETERMINANTS OF PDLIM2 IN SUPPRESSING HTLV-I TAX-MEDIATED TUMORIGENESIS

Portions of the research in this chapter are currently in press at *Oncogene* with authors Jing Fu, Pengrong Yan, Shirong Li, Zhaoxia Qu and Gutian Xiao.

3.1 SUMMARY

Human T cell leukemia virus type I (HTLV-I) encodes a Tax oncoprotein that plays a crucial role in both viral replication and cell transformation. Our studies found that PDLIM2 negatively regulates Tax by promoting the proteasomal degradation of Tax, thereby suppresses the transformative ability of HTLV-I. The counterbalance between HTLV-I/Tax may determine the outcome of HTLV-I infection. Here we further showed that the interaction between PDLIM2 and Tax was mediated by a putative α-helix motif within PDLIM2 at amino acids 236-254. PDLIM2 with selective disruption of this short helix failed to promote Tax degradation and lost the ability in tumor suppression. Although the C-terminal LIM domain of PDLIM2 was not required for Tax binding, it is required to trigger the poly-ubiquitination of Tax and determine Tax subcellular distributions. In contrast, the N-terminal PDZ domain of PDLIM2 was largely dispensable. These studies dissect functional sequences within PDLIM2 and their distinct roles in HTLV-I Tax regulation.

3.2 INTRODUCTION

HTLV-I is an oncogenic retrovirus etiologically associated with adult T cell leukemia (ATL) (Grassman *et al*., 2005; Sun and Yamaoka, 2005). The virus encodes a 40-kDa regulatory protein named Tax. Tax protein is not only required for virus replication but is also able to immortalize many different cells including human primary T cells (Grassmann *et al*., 1992; Pozzatti *et al*., 1990; Tanaka *et al*., 1990). In addition, Tax-transformed cells induce tumors when introduced into nude or SCID mice (Oka *et al*., 1992; Pozzatti *et al*., 1990). More importantly, the HTLV-I genome without Tax loses its original transformative ability (Yamaoka *et al*., 1992), whereas Tax-transgenic mice develop various tumors, depending on the type of promoter used to drive Tax expression (Grossman *et al*., 1995; Nerenberg *et al*., 1987; Peebles *et al*., 1995). Indeed, Tax-immortalized lymphocytes *in vitro* and Tax-mediated T cell lymphomas in animal closely resemble the phenotypes of HTLV-I-transformed T cells and HTLV-I-induced ATL, respectively (Akagi *et al*., 1995; Hasegawa *et al*., 2006; Kwon *et al*., 2005).

Tax oncoprotein exerts its oncogenic role largely through deregulation of cellular transcription factors that are critical for cell growth and division, such as NF-κB (Grassman *et al*., 2005; Sun and Yamaoka, 2005). In the cytosol, Tax recruits the IκB (inhibitor of NF-κB) kinase (IKK) complex into specific perinuclear structures for IKK activation, resulting in the degradation of IκB and the subsequent nuclear translocation of NF-κB factors, including p65, the prototypic member of NF-κB (Xiao *et al*., 2006). In the nucleus, Tax recruits p65 and other

cellular transcriptional components into inter-chromatin granules to form discrete transcriptional hot-spots named ‘Tax nuclear bodies’ or ‘Tax nuclear foci’ (Bex *et al*., 1997; Semmes and Jeang, 1996).

Although the mechanisms by which Tax hijacks cellular signaling for its oncogenic action have been extensively investigated, the molecular studies on how HTLV-I Tax is regulated by cellular factors are still lacking. We characterize PDLIM2, a newly identified PDZ-LIM domain-containing protein with ubiquitination promoting activity, as an intrinsic negative regulator of Tax (Yan *et al.*, 2009).

As the name suggests, the PDLIM2 protein consist of both PDZ and LIM domains. The LIM domain is responsible for the E3 ligase functions of PDLIM2 whereas the PDZ domain involves in protein intra-nuclear trafficking (Tanaka *et al.*, 2007). Citing p65 as an example, the deletion of the p65 LIM domain induced a dramatic decrease in p65 poly-ubiquitination (Tanaka *et al.*, 2007). However, a lack of PDZ domain results in the insufficient degradation of p65 with neither decreased nuclear-soluble p65 nor increased nuclear-insoluble p65 (Tanaka *et al.*, 2007), suggesting that intra-nuclear trafficking of p65 is necessary for its degradation. Additionally, mutant PDLIM2 proteins harboring a deletion of its LIM or PDZ domains still retain the ability to interact with p65. Although the author of this study explained that more than one region within PDLIM2 is required for the PDLIM2-p65 interaction, we cannot exclude the possibility that the region between LIM and PDZ is essential for the PDLIM2-p65 interaction.

We have found that PDLIM2 functions as an intrinsic negative regulator of HTLV-I Tax. It directly bound to Tax, formed a stable complex, promoted Tax poly-ubiquitination and subsequent proteasomal degradation (Yan *et al.*, 2009). Accordingly, co-expression of PDLIM2

suppressed Tax mediated downstream signaling and tumorigenicity (Yan *et al.*, 2009). Even more importantly, re-introduction of PDLIM2 into HTLV-I-transformed T cells blocks their tumorigenicity in mice (Yan *et al.*, 2009). These findings make PDLIM2 the first intrinsic cellular factor that regulates the oncoprotein Tax directly and negatively (Yan *et al.*, 2009). It is therefore interesting and important to address the molecular determinants within both PDLIM2 and Tax, which might be responsible for their biological functions.

3.3 MATERIALS AND METHODS

Expression vectors and reagents

Expression vectors encoding Tax, PDLIM2, PDLIM2 ΔPDZ and PDLIM2 ΔLIM mutants have been described before (Qu *et al.*, 2004; Tanaka *et al.*, 2007). Other PDLIM2 mutants mentioned in this section were generated via mutagenesis PCR using primers in Appendix B. Ubiquitin construct was gifts of Dr. Z. Chen. GFP-Tax and its mutants were gifts of Dr. O. J. Semmes. cDNAs of Tax, PDLIM2 and PDLIM2 mutants were also sub-cloned into retroviral vectors pCLXSN and/or pTRIP by routine cloning strategies as described (Qing *et al.*, 2005a). The HA monoclonal antibody (12CA5) and HRP-conjugated HA monoclonal antibody (3F10) were from Roche Molecular Biochemicals. The ubiquitin, Sp1, lamin B, Hsp90, and β-actin antibodies as well as the pre-immune IgG were from Santa Cruz Biotechnology. The proteasome inhibitor MG132 and protein synthesis inhibitor cycloheximide (CHX) were from Biomol. Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Sigma. The Tax and MYC antibodies were described previously (Qu *et al.*, 2004).

Cell culture and transfection

HEK293 cells and Rat-1 were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 2 mM L-glutamine (Invitrogen), and HTLV-I-transformed T cell lines C8166 were maintained in suspension in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Yan *et al.*, 2007). 293 were transfected with DEAE-Dextran (Sigma) (Qing and Xiao, 2005; Xiao and Sun, 2000).

Retroviral transduction and generation of stable transfectant

Rat-1 cells were infected with virus expressing Tax or PDLIM2, respectively. The Rat-1 cells stably expressing Tax were also re-infected with virus expressing PDLIM2 or PDLIM2 mutants for simultaneously expressing both Tax and PDLIM2. The viruses expressing GFP were used as a control. The stable transfectants were obtained by selection with G418 and/or blasticidin selections as described previously (Qing *et al.*, 2007).

Subcellular fractionation, immunoblotting (IB) and immunoprecipitation (IP) assays

Cytoplasmic, soluble and insoluble nuclear extracts were prepared using the hypotonic buffer (20 mM HEPES, pH 8.0, 10 mM KCl, 1 mM $MgCl_2$, 0.1% (vol/vol) Triton X-100 and 20% (vol/vol) glycerol), hypertonic buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 20% (vol/vol) glycerol, 0.1% (vol/vol) Triton X-100 and 400 mM NaCl), and insoluble buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% (wt/vol) SDS, 1% (vol/vol) NP-40 and 10 mM iodoacetamide), respectively (Tanaka *et al.*, 2007). The purity of the obtained fractions was confirmed by

checking Hsp90 (cytoplasm), Sp1 (soluble nuclear fraction), or lamin B (insoluble nuclear fraction). Total nuclear extracts were prepared by simply lysing pellets in insoluble buffer after the cytoplasm was extracted. Whole-cell extracts were prepared by lysing cells in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 0.25% (wt/vol) Na-deoxycholate, 1% (vol/vol) NP-40, 1 mM DTT). All the lysis buffers were supplemented with 1 mM PMSF and a protease inhibitor cocktail (Roche Molecular Biochemicals). The cells extracts were used for IP and/or IB assays as described before (Xiao *et al.*, 2001a).

In vivo ubiquitin conjugation assay

Protein extracts were prepared from 293 cells transfected with Tax together with HA-tagged ubiquitin in the presence or absence of PDLIM2, immediately followed by IP using anti-Tax. The ubiquitin-conjugated Tax pulled down by IP was detected by IB using anti-HA-HRP (Xiao *et al.*, 2001b).

Protein stability assay

Cells were treated with 10 μM CHX, followed by chasing of the indicated time period in the presence or absence of MG132, and IB to detect the indicated proteins (Qing *et al.*, 2005b).

MTT cell proliferation assay

Cells were cultured under normal growth medium and subjected to MTT treatment (Final concentration 0.5mg/mL) for 4hrs. The cells were dissolved completely using the converted dye (0.04M HCl in absolute isopropanol). After a brief centrifuge, absorbance of the converted dye was measured at a wavelength of 560nm with background subtraction at 670nm using Beckman DU-600 Spectrophotometer.

Colony formation assays

Soft agar assays were performed as previously described (Qing *et al.*, 2007). Briefly, 6-well plates were coated with an initial underlay of 1% SeaPlaque low melting agarose in culture medium. Cell suspension in culture medium containing 0.6% SeaPlaque low melting agarose was then added to the coated plates. Colony growth was scored after 21 days of cell incubation at the normal condition. All the colony formation assays presented in this study were repeated in at least three independent experiments, and each independent experiment included three different cell doses: 5×10^3, 1×10^4, and 5×10^4.

In vivo tumorigenicity assays

Five-week old female SCID mice (C.B-17^{TM}/IcrCrl-scidBR) (from Charles River Lab.) were challenged subcutaneously in hind-back with Rat-1 stable cell lines. The recipient mice were monitored, sacrificed and dissected for tumor evaluation at the indicated post-injection days.

Statistical significance

Paired t-test for independent samples was used to assess statistical significance.

3.4 RESULTS

3.4.1 Both the C-terminal LIM domain and the internal motif 236-254 are required for PDLIM2-induced Tax degradation.

3.4.1.1 The internal 19 amino-acid motif 236-254 is required for the interaction between PDLIM2 and Tax.

Tax contains a PDZ-domain-binding motif (PBM) at its C-terminus, which is responsible for its interaction with various other PDZ-domain-containing proteins, including DLG1 (Lee *et*

al., 1997; Rousset *et al.*, 1998). We initially tested the role of the Tax PBM and PDZ domain of PDLIM2 through PDLIM2-Tax interaction assays.

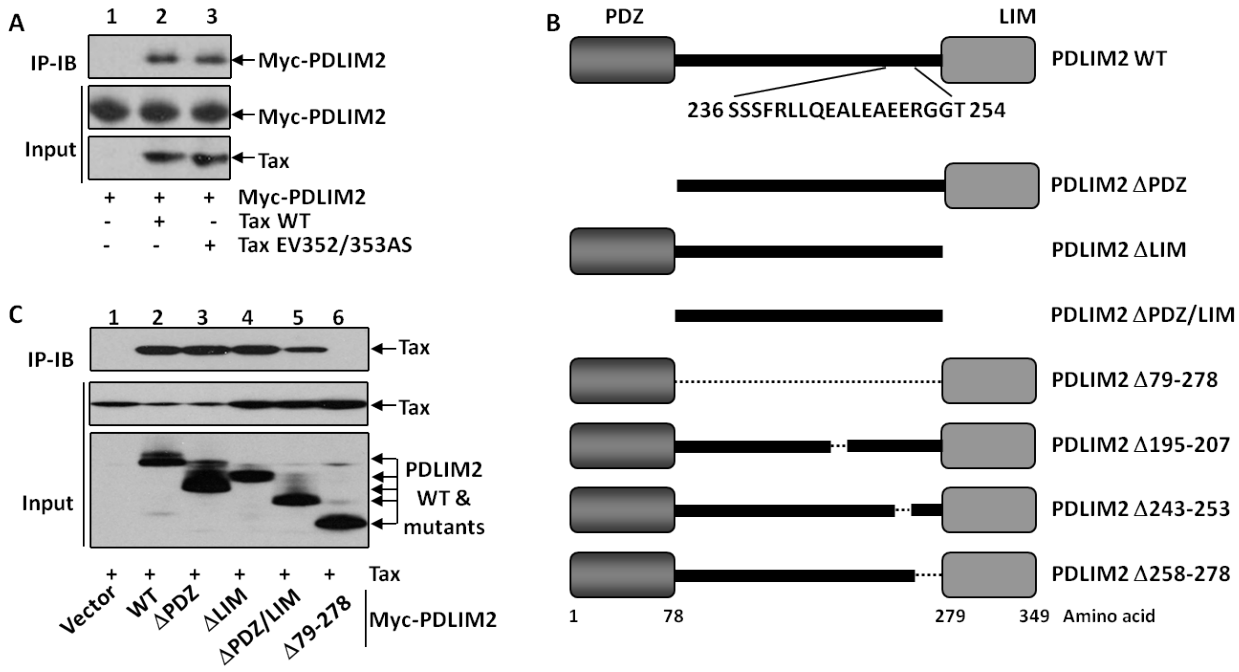

Figure 20. Interaction between PDLIM2 and Tax is independent of the Tax PBM domain and the PDZ domain of PDLIM2.

(A) 293 cells were transfected with myc-PDLIM2 alone or together with Tax WT or Tax EV352/353AS, the PBM disruption mutant. Lysates from the indicated cells were subjected to IP assays using Tax antibody and IB by myc antibody. The inputs of myc-PDLIM2, Tax and Tax EV352/353AS were analyzed by direct IB. (B) Schematic representation of PDLIM2 and its mutants used in this study. Amino acid 236-254, a putative short α-helix that acts as the Tax binding motif of PDLIM2, is also shown. (C) Lysates from 293 cells expressing Tax alone or in the presence of the indicated PDLIM2 internal deletion mutants were analyzed by IP using myc antibody and by IB using Tax antibody.

Surprisingly, disruption of the PBM motif within Tax (Tax EV352/353AS) failed to block the Tax-PDLIM2 interaction (Figure 20A). Consistently, deletion of the PDZ domain of

PDLIM2 (ΔPDZ) did not affect the interaction with Tax either, indicating that the PDLIM2-Tax interaction is independent of the Tax PBM and the PDZ domain of PDLIM2 (Figure 20C). Similarly, deletion of the C-terminal LIM domain (ΔLIM) had no effect on Tax binding (Figure 20C). Moreover, the simultaneous deletion of both PDZ and LIM domains (ΔPDZ/LIM) showed the binding capacity (Figure 20C). In contrast, sequences between the PDZ and LIM domains (Δ79-278), which is commonly considered as a disordered region, present to be indispensable and responsible for the interaction between PDLIM2 and Tax (Figure 20C). Therefore, the sequences covering the middle region between the PDZ and LIM domains may contain a Tax-binding motif.

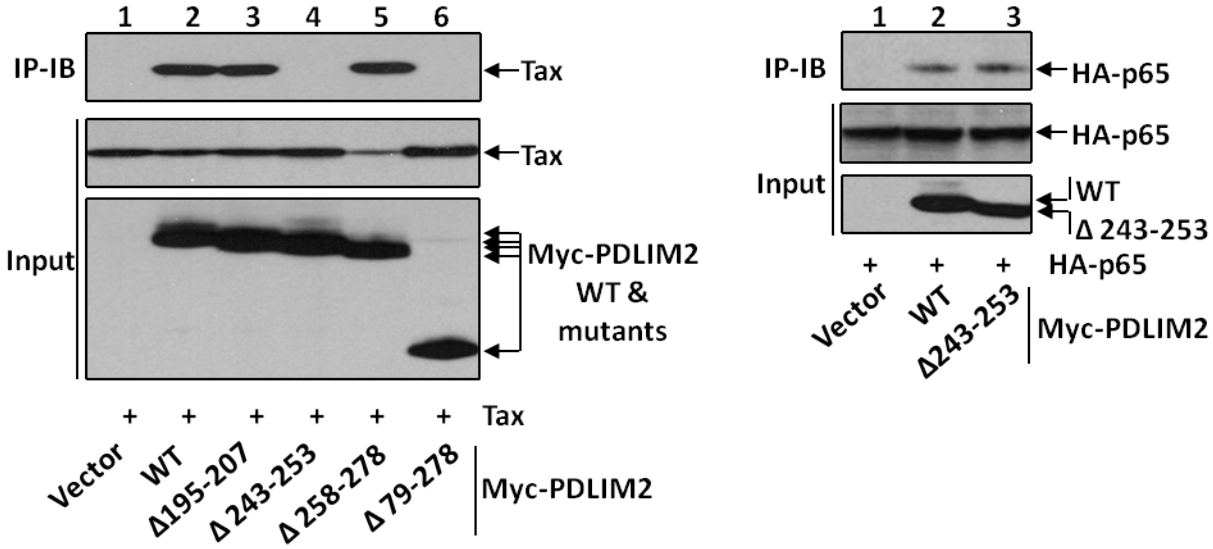

Figure 21. Sequences at 243-253 are required for PDLIM2-Tax interaction.

(A) Lysates from 293 cells expressing Tax alone or in presence of the indicated PDLIM2 internal deletion mutants were analyzed by IP using myc antibody and by IB using Tax antibody. (2) Similar IP assay were performed by transfecting 293 cells with HA-p65 alone or together with myc-PDLIM2 WT or myc-PDLIM2 D243-253 to address the interaction between PDLIM2-p65.

To characterize the Tax-binding motif within PDLIM2, we generated various small internal deletion mutants of PDLIM2 (Figure 20B). Interestingly, internal deletion of amino acids 243-253 (Δ243-253) caused complete loss of the PDLIM2-Tax interacting ability (Figure 21A). The loss of Tax binding capacity seemed not due to structural disruption since the mutants with deletions of the sequences ahead of or behind the motif (Δ195-207 and Δ258-278) did not affect the PDLIM2-Tax interaction (Figure 21A). Importantly, deletion of the amino acids 243-253 did not affect PDLIM2 binding to p65, another primary target of PDLIM2 (Figure 21B), indicating that the amino acids 243-253 are required specifically for the Tax binding.

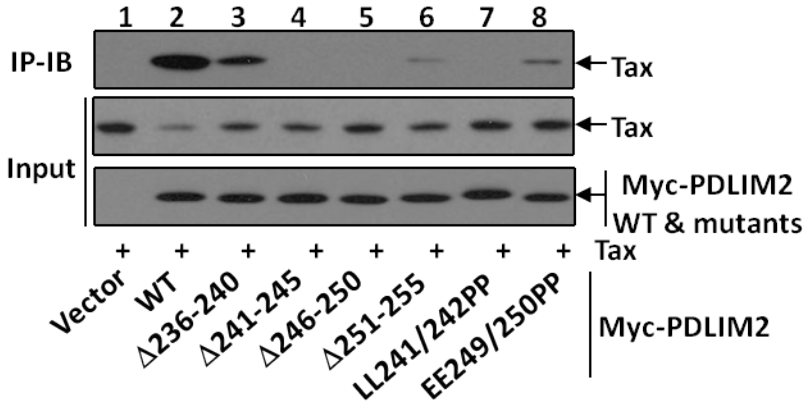

Figure 22. The putative α-helix 236-254 is required for PDLIM2-Tax interaction.

Lysates from 293 cells expressing Tax alone or in presence of the indicated PDLIM2 deletion or substitution mutants were analyzed by IP using myc antibody and by IB using Tax antibody.

Further analysis of the primary structure of PDLIM2 showed that the amino acids 243-253 might locate within a putative α-helix motif of PDLIM2 covering amino acids 236-254 (Figure 20B, www.predictprotein.org). In accordance, it is reported that the short helix is the preferential structure for Tax binding (Xiao *et al.*, 2000). To test whether this short α-helix was responsible for the PDLIM2-Tax interaction, we disrupted the helix by smaller internal deletions

or point substitutions with prolines. As shown in Figure 22, disruption of the short helix, by its middle part small deletion (Δ241-245 or Δ246-250) or leucine to proline substitutions (LL241/242PP), abolished the PDLIM2-Tax interaction, although mutations at the ends of the short helix (Δ236-240 or Δ251-255, or EE249/250PP) led to a partial loss of binding capacity. Taken together, the results suggested that the putative α-helix motif of PDLIM2 is the Tax-binding motif required for the PDLIM2-Tax interaction.

3.4.1.2 Different sequences within Tax are involved in binding to PDLIM2.

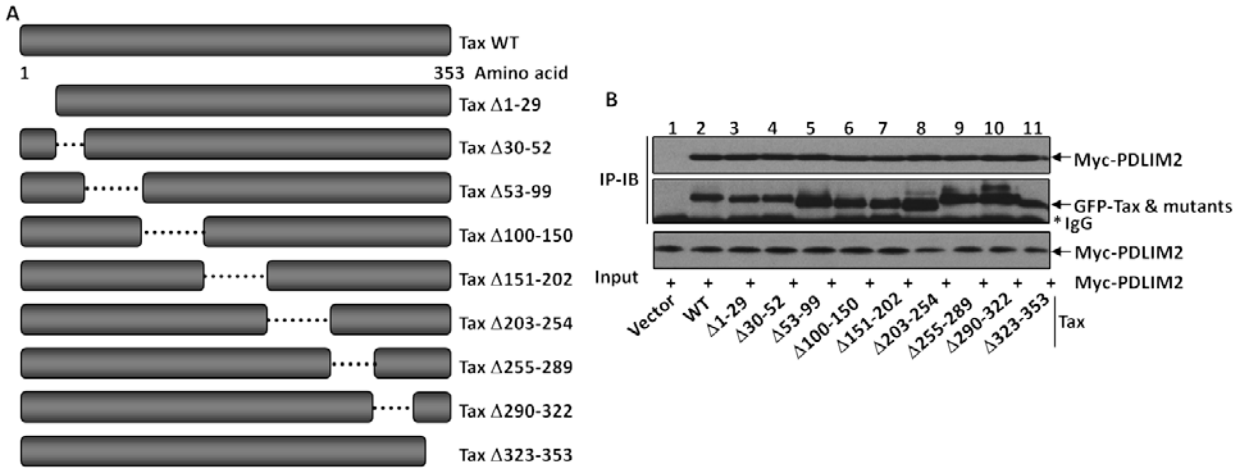

Figure 23. Different sequences within Tax are involved in interaction with PDLIM2.

(A) Schematic representation of Tax-GFP and its mutants. The fused GFP epitope tag was not shown. (B) Lysates from 293 cells transfected the indicated constructs were subjected to IP assays using GFP antibody and IB using myc antibody. Membrane was stripped and re-blotted for detection of pulled down GFP-Tax. The input of myc-PDLIM2 was detected by direct IB using myc antibody.

In order to identify the PDLIM2-binding motif within Tax, we took advantage of the deletion mutants of Tax that systematically cover the whole sequences of Tax (Figure 23A (Fryrear *et al.*, 2009). Interestingly, all Tax mutants, like the WT protein, still retained the

capacity to bind PDLIM2 (Figure 23B), indicating that more than one motif within Tax can complementarily interact with PDLIM2. This data is consistent with the fact that Tax is an intrinsically disordered protein (IDP), which contains typical disordered regions (Boxus *et al.*, 2008).

3.4.1.3 Both the C-terminal LIM domain and the Tax-binding motif (TBM) are required for Tax poly-ubiquitination and degradation by PDLIM2.

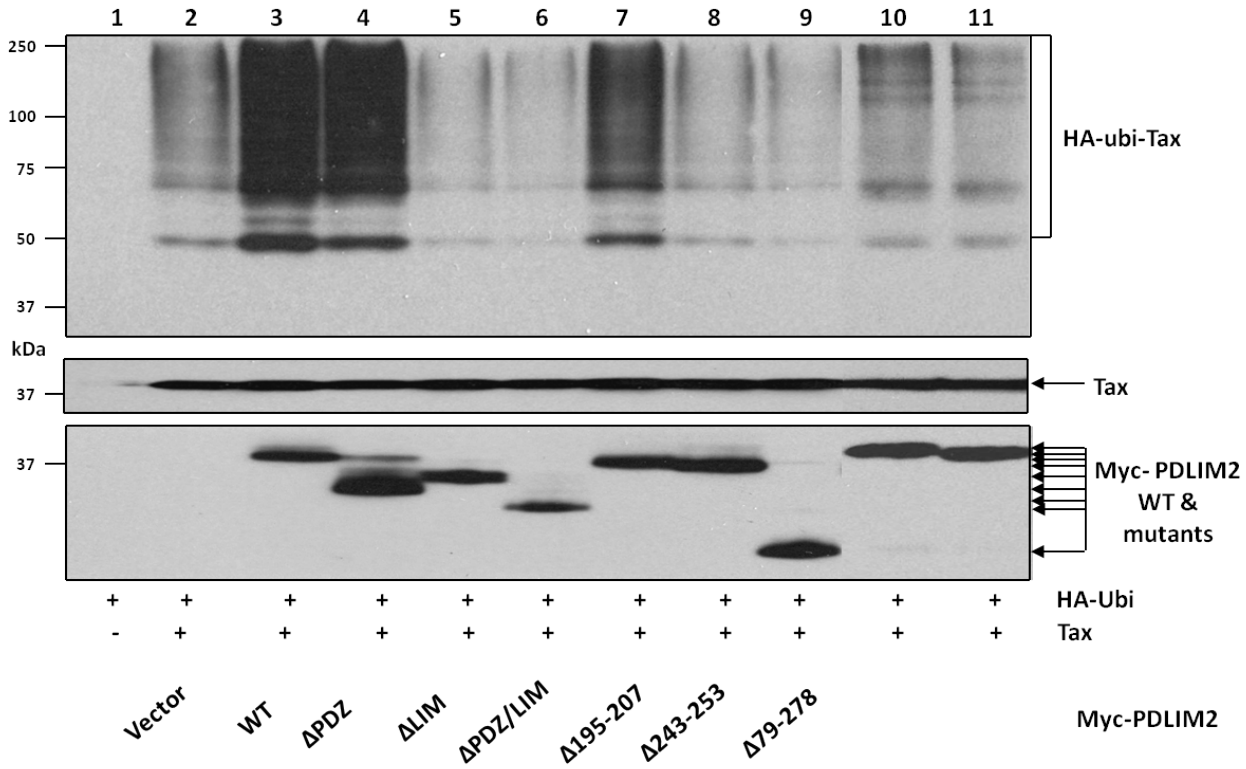

Figure 24. Both the LIM domain and the Tax-binding motif (TBM) within PDLIM2 are required for promoting Tax poly-ubiquitination.

293 cells were transfected with the indicated constructs and subjected to the treatments with MG132 for 16hrs, followed by nuclear extractions as described. IP assays were performed using Tax antibody for pulling down and IB using HA-HRP antibody. Same membrane was stripped and re-blotted for detection of pulled down Tax. The inputs of myc-PDLIM2 were analyzed by IB.

As described in the former chapter, PDLIM2 induced Tax repression involves promoted Tax poly-ubiquitination and the subsequent proteasomal degradation. Thus, we characterized the distinct functions of indicated PDLIM2 mutants in regulation of Tax poly-ubiquitination and degradation. Consistent with previous studies, WT PDLIM2 promoted Tax poly-ubiquitination (Figure 24). In agreement with the ubiquitin E3 ligase function of the LIM domain within PDLIM2, the ΔLIM or ΔPDZ/LIM mutant of PDLIM2 failed to promote Tax poly-ubiquitination (Tanaka *et al.*, 2007; Tanaka *et al.*, 2005). Similarly, all PDLIM2 mutants with deletion or mutation of Tax binding motif (TBM) such as the Δ79-278, Δ243-253, LL241/242PP and EE249/250PP mutants lost their ability to promote Tax poly-ubiquitination (Figure 24). In contrast, the ΔPDZ and Δ195-207 mutants, that maintained the full LIM domain and TBM, kept the ability to promote Tax poly-ubiquitination. These results suggested that elevated Tax poly-ubiquitination requires both LIM domain and TBM within PDLIM2.

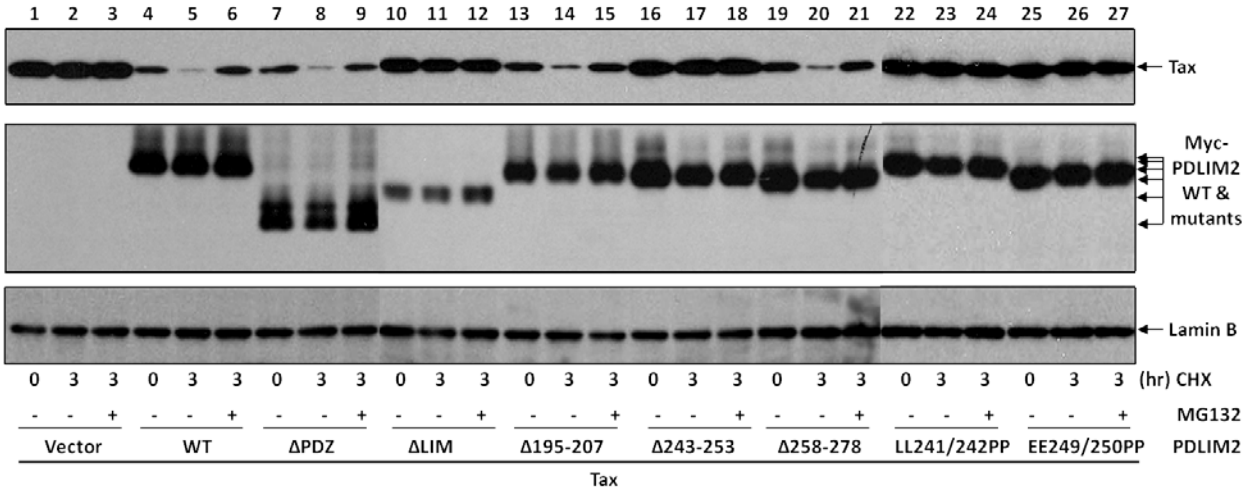

Figure 25. Both LIM domain and TBM within PDLIM2 are required for promoting Tax turnover.

293 cells transfected with the indicated constructs were mock-treated or treated with CHX in the presence or absence of MG132 for 3hrs. The nuclear insoluble fractions were utilized for IB using Tax and myc antibody. Lamin B was detected as the protein loading control.

In agreement with the above findings, we further confirmed that the PDLIM2 mutants lacking of a functional LIM or TBM domains (ΔLIM, Δ243-253, LL241/242PP, EE249/250PP) failed to promote Tax turnover in CHX-chase assay (Figure 25). Thus, both LIM domain and TBM of PDLIM2 are crucial for the proteasomal degradation of Tax, although they played distinct roles in the events.

Since PDLIM2 ΔLIM mutant binds to Tax but does not induce Tax degradation, we thought interesting to examine whether this mutant can function as a dominant-negative form of PDLIM2. To this effect, we generated C8166 cells stably expressing an empty vector, PDLIM2 WT or PDLIM2 ΔLIM, as this HTLV-I-transformed T cell line expresses a modest level of endogenous PDLIM2 (Figure 30). Consistently, Tax protein underwent a modest proteasomal degradation, which was significantly accelerated by stable expression of exogenous PDLIM2 (Figure 26). As expected, the Tax degradation was largely blocked by expression of PDLIM2 ΔLIM mutant. These results indicate that PDLIM2 ΔLIM can function as a dominant-negative form of PDLIM2, further supporting the distinct roles of the LIM domain and TBM in PDLIM2-mediated Tax degradation.

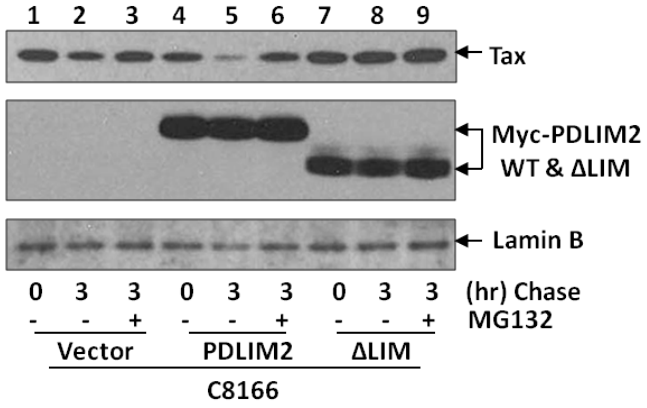

Figure 26. PDLIM2 mutant with deletion of the LIM domain serves as a dominant-negative form of PDLIM2.

HTLV-I-transformed T cell line C8166 stably expressing PDLIM2 WT, PDLIM2 ΔLIM or an empty vector were subjected to CHX-Chase assays as described before.

3.4.1.4 Both the LIM domain and the TBM are responsible for Tax subcellular redistribution.

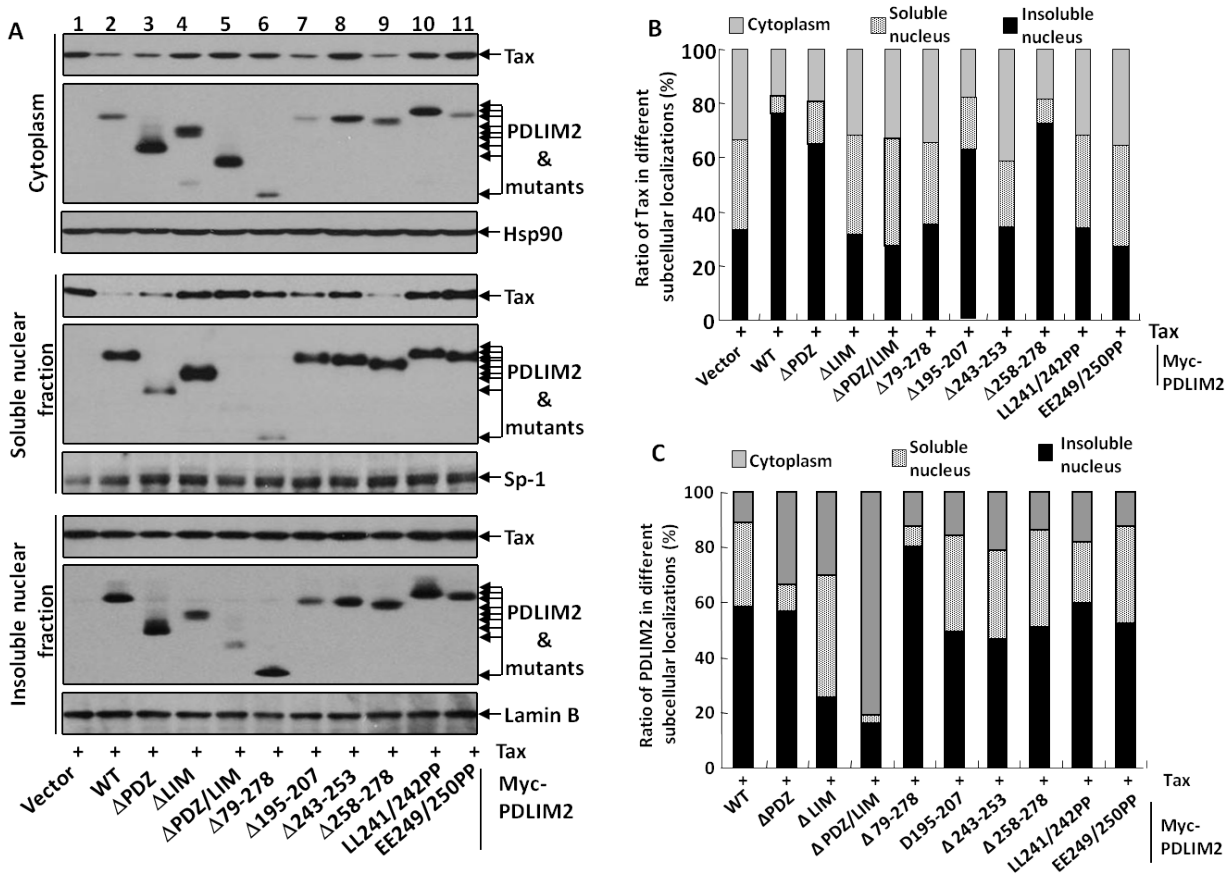

Figure 27. Both the LIM domain and the TBM within PDLIM2 are required for Tax subcellular redistribution.

(A) Effect of different sequences within PDLIM2 on Tax subcellular expression. 293 cells were transfected with Tax alone or together with the indicated PDLIM2 constructs, followed by cell fractions and IB to detect expression levels of the indicated proteins. (B) Quantitation of Tax protein in different subcellular fractions. The Tax bands in A were quantitated by densitometry and the ratios of Tax in the cytoplasmic, soluble nuclear and insoluble nuclear

fractions to total Tax were presented in percentile. (C) Quantitation of PDLIM2 protein in different subcellular fractions. The PDLIM2 bands in A were quantitated by densitometry and the ratios of PDLIM2 in the cytoplasmic, soluble nuclear and insoluble nuclear fractions to total PDLIM2 were presented in percentile.

To tracking the location of Tax in the cells, we did fraction assays to measure Tax protein levels in the cytoplasmic, soluble nuclear, and insoluble nuclear fractions using 293 cells which expressed undetectable endogenous PDLIM2 protein (Figure 30). As shown in Figure 28, Tax was expressed in all three fractions in the absence of PDLIM2, while expression of PDLIM2 resulted in a significant increasing of Tax ratio into the nuclear insoluble fraction, together with a decrease of Tax total protein.

Similar to the PDLIM2 WT, PDLM2 mutants deleting the PDZ domain (ΔPDZ), amino acids 195-207 (Δ195-207) and 258-278 (Δ258-278) were able to affect the Tax subcellular distribution (Figure 27). However, PDLIM2 mutants with deletion of the LIM domain (ΔLIM and ΔPDZ/LM) and mutants without the full Tax-binding motif (Δ79-278, Δ243-253, LL241/242PP and EE249/250PP) lost this ability (Figure 27). In summary, the results indicated that both LIM domain and the TBM, but not the PDZ domain within PDLIM2 were responsible for determining Tax subcellular distribution, which is in agreement with the biological function of PDLIM2 to trigger proteasomal degradation of Tax.

3.4.2 Both the LIM domain and the PDLIM2-Tax interaction are involved in PDLIM2 mediated suppression of Tax tumorigenicity

3.4.2.1 Deletion of the LIM domain and disruption of TBM failed to block Tax mediated anchorage-independent colony formation.

To further investigate the significance of these domains in PDLIM-mediated suppression of Tax tumorigenicity, we generated Rat-1 fibroblasts stably expressing Tax alone or together with different PDLIM2 mutants. Although the expression of Tax alone or together with PDLIM2 WT or PDLIM2 mutants had no or minor effect on the growth of Rat-1 cells in normal culture condition (Figure 28A), Tax induced anchorage-independent colony formation of Rat-1 cells in soft agar (Figure 28B). The WT PDLIM2 was able to prevent the colony growth, while either deletion of the LIM domain (ΔLIM) or disruption of TBM (Δ243-253, LL241/242PP and EE249/250PP) resulted in loss of this suppression ability (Figure 28B). In contrast, deletions of the PDZ domain (ΔPDZ) or the amino acids 258-278 (Δ258-278) had no statistically significant effects on the suppression of Tax-mediated Rat-1 cell transformation (Figure 28B). Importantly, the results are correlated with the functions of domains in promoting Tax turnover and Tax proteasomal degradation.

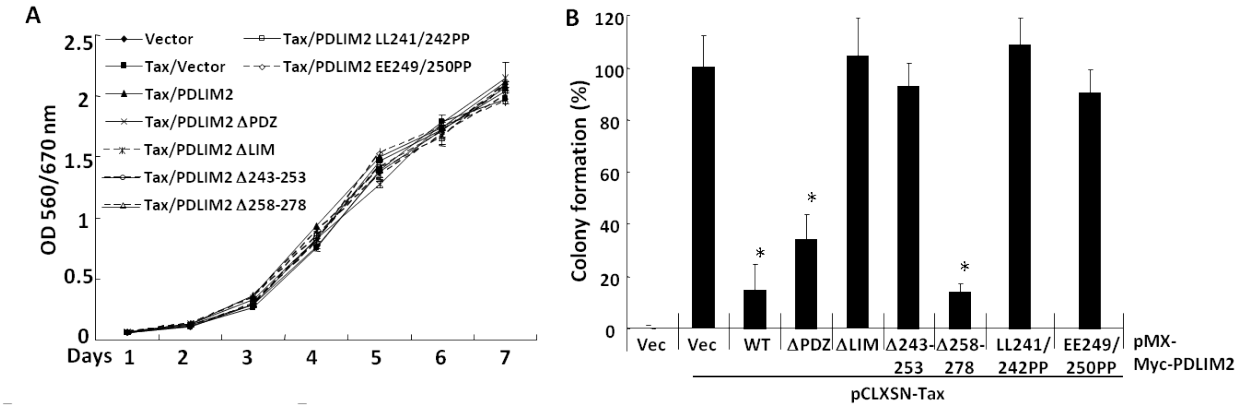

Figure 28. Deletion of the LIM domain or disruption of TBM of PDLIM2 fails to suppress Tax-mediated colony formation.

(A) Rat-1 stable cell lines cultured under normal growth medium were subjected to MTT assay to determine the live cells. Proliferation curve was generated based a collection of constitutive 7 days culturing and measuring. (B)The indicated Rat-1 stable cells were plated in soft agar for colony formation. Colony numbers were counted at day 21 after plating. The data presents the percentile of colony formation compared to the cells expressing Tax alone (set as 100). Error bars indicate standard deviations (n = 3).

3.4.2.2 Deletion of the LIM domain or the disruption of TBM fails to suppress Tax-mediated tumor growth in SCID mice.

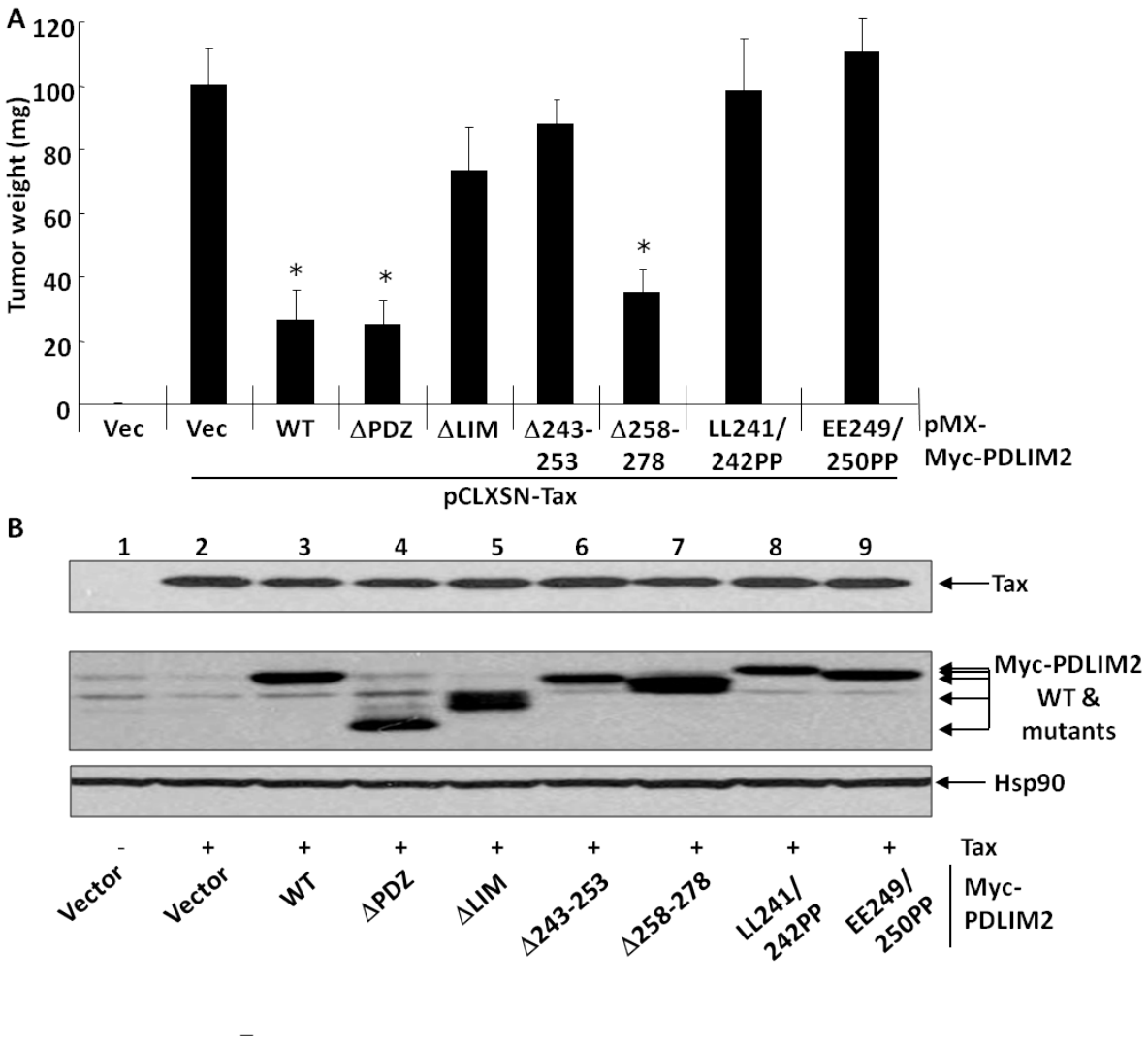

Figure 29. Deletion of the LIM domain or disruption of TBM of PDLIM2 fails to suppress Tax mediated tumor growth in SCID mice.

(A) The indicated Rat-1 stable cells were subcutaneously inoculated into the SCID mice. The mice were sacrificed at day 14 after inoculation and tumor weights were measured. The data presented are the mean ± standard deviation (n≥ 3). (B) Expression levels of Tax and Myc-PDLIM2 constructs in the stable cells were examined by direct IB using the whole cell lysates. The expression of endogenous Hsp90 was used as a loading control.

To further confirm these results *in vivo*, we subcutaneously injected those Rat-1 cells into SCID mice. In agreement with Figure 13, expression of WT PDLIM2 repressed Tax-mediated xenograft growth (Figure 29). Defects of PDLIM2 either in the LIM domain or the TBM, but not the PDZ domain or amino acids 258-278, lost such suppressive capacity (Figure 29), indicating that both of them were required to suppress Tax-mediated tumorigenesis in the SCID mice.

3.5 DISCUSSION

Our studies demonstrated that PDLIM2 promotes Tax poly-ubiquitination and proteasomal degradation and serves as an intrinsic negative regulator of HTLV-I Tax to affect the Tax-mediated signal transduction and tumorigenesis. Here we further dissected different functional sequences within PDLIM2.

A putative α-helix motif spanning amino acids 236-254 of PDLIM2 was defined as the molecular determinant for PDLIM2-Tax interaction. Selective disruption of this short helix crippled PDLIM2 functions in determining Tax subcellular distribution and subsequent ubiquitination and proteasomal degradation, resulting in the defect of PDLIM2 in tumor suppression. However, both the C-terminal LIM domain and the N-terminal PDZ domain, which are predicted to be the major functional domains of PDLIM2, were dispensable for Tax binding (Te Velthuis and Bagowski, 2007; Te Velthuis *et al.*, 2007). In further support of the ubiquitin E3 ligase function of LIM domain, loss of the LIM domain leads to the failure of PDLIM2-mediated Tax repression.

These studies provide detailed insights into the molecular actions of PDLIM2 on Tax regulation. Furthermore, they also have the general significance in cancer biology and treatment, given our recent findings linking PDLIM2 epigenetic repression to pathogenesis of different cancers such as breast cancer and colon cancer (Qu *et al.*, 2009a; Qu et al, 2009b). More detailed discussion will be presented in the Overall Discussion.

4.0 HTLV-I-MEDIATED REPRESSION OF PDLIM2 INVOLVES DNA METHYLATION, BUT INDEPENDENT OF TAX

Work described in this chapter was published in *Neoplasia* with authors Pengrong Yan, Zhaoxia Qu, Chie Ishikawa, Naoki Mori and Gutian Xiao. (*Neoplasia.* 2009, 11(10):1036-41.)

4.1 SUMMARY

Human T cell leukemia virus type I (HTLV-I) is the etiological agent of adult T cell leukemia (ATL). We found that HTLV-I–mediated tumorigenesis involves the repression of PDLIM2. PDLIM2 expression levels remains low in primary ATL cells freshly isolated from patients as well as established ATL cell lines. Moreover, such HTLV-I–mediated PDLIM2 repression may be a pathophysiological event associated with the long latency of ATL progression. Our results also suggest that the PDLIM2 repression involved DNA methylation. Whereas DNA methyltransferase (DNMT) 1 and 3b, but not 3a, were up-regulated in HTLV-I–transformed T cells, the DNMT inhibitor 5-aza-dC restored PDLIM2 expression and induced death of these malignant cells. We found that the PDLIM2 repression was independent of the HTLV-I Tax because neither short-term induction nor long-term stable expression of Tax affects PDLIM2 expression. These studies not only provide important insights into the involvement of DNA methylation in Tax regulation and HTLV-I leukemogenicity by PDLIM2, but also suggest that DNMT inhibitor, 5-aza-dC, shows potential therapeutic capacity for ATL treatment.

4.2 INTRODUCTION

Human T cell leukemia virus type I (HTLV-I) infection causes an aggressive and fatal $CD4^+$ T cell malignancy termed adult T cell leukemia (ATL) in approximately 2-5% virus carriers after an extensive latency period. Although the detailed mechanisms of ATL development remain under investigation, it seems clear that the early stage of HTLV-I-mediated leukemogenesis is largely mediated by its encoded regulatory protein Tax. Tax is able to transform many different types of cells *in vitro* and induce tumors in mice. Conversely, the HTLV-I genome without Tax loses its original oncogenic ability.

The Tax oncoprotein exerts its transforming action largely through deregulation of cellular transcription factors that are critical for cell growth and division, such as nuclear factor κB (NF-κB) (Grassmann *et al.*, 2005; Sun and Yamaoka, 2005). The mechanisms by which Tax deregulates the NF-κB signaling for tumorigenesis have been extensively studied. In the cytoplasm, Tax recruits the inhibitor of NF-κB (IκB) kinase (IKK) complex into specific compartments for IKK activation (Harhaj *et al.*, 2007; Kfoury *et al.*, 2008), resulting in the degradation of IκB and the subsequent nuclear translocation of NF-κB factors, including p65 (Xiao *et al.*, 2006). In the nucleus, Tax recruits p65 and other cellular transcriptional components into inter-chromatin granules to form discrete transcriptional hot spots termed as Tax nuclear bodies for full NF-κB transcriptional activation (Bex *et al.*, 1997; Semmes and Jeang, 1996).

Unlike the mechanisms by which Tax hijacks cellular signaling to initiate malignant transformation leading to the development of ATL, the molecular studies on how HTLV-I/Tax is regulated by cellular factors are still lacking. We have identified PDLIM2 as an intrinsic negative regulator of Tax (Yan *et al.*, 2009). PDLIM2 not only promotes Tax poly-ubiquitination but also binds to and shuttles Tax from its functional sites including the perinuclear structures and Tax nuclear foci for proteasomal degradation. Accordingly, PDLIM2 prevents downstream signaling and subsequent tumorigenicity of HTLV-I/Tax. Interestingly, PDLIM2 is conversely repressed in various HTLV-I-transformed cells at the transcriptional level. These studies suggested that PDLIM2 repression is one important mechanism of HTLV-I-mediated tumorigenesis. However, whether the PDLIM2 repression occurs under pathophysiological conditions and how HTLV-I represses PDLIM2 expression still remain unknown.

We further reported that PDLIM2 expression levels remains low in primary ATL cells freshly isolated from patients as well as established ATL cell lines. However, the PDLIM2 repression was independent of the viral oncoprotein Tax because neither short-term induction nor long-term stable expression of Tax down-regulated PDLIM2 expression. Instead, the HTLV-I-mediated PDLIM2 repression involved DNA methylation. Whereas DNMT 1 and 3b, but not 3a, were up-regulated in HTLV-I–transformed T cells, the DNMT inhibitor, 5-aza-dC, reactivated PDLIM2 expression. In agreement with that, we found that 5-aza-dC was able to induce death of HTLV-I-transformed T cells in a dose-dependent manner. These studies not only provide important insights into PDLIM2 regulation and HTLV-I leukemogenicity, but also suggest potential therapeutic strategies for ATL by using 5-aza-dC.

4.3 MATERIALS AND METHODS

Reagents

Tet-free fetal bovine serum (FBS), doxycycline, and 5-aza-2'-deoxycytidine (5-aza-dC) were from Hyclone Laboratories (Logan, UT) and Sigma (St. Louis, MO), respectively. PDLIM2 antibodies were gift of Dr. R. O'Connor (Loughran *et al.*, 2005; Tanaka *et al.*, 2005).

Cell culture and transfection

Human T lymphocyte Jurkat, and HTLV-I-transformed T cell lines C8166, HuT102, MT-4, SLB-1 and TL-Om1 were maintained in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Yan *et al.*, 2007). Jurkat Tet-on cells with inducible Tax were cultured with 10% Tet-free FBS and 2mM L-glutamine in DMEM and RPMI 1640 medium.

Induction of Tax or PDLIM2 in Jurkat-TetOn-inducible cell line

For Tax induction, Jurkat-TetOn-inducible cells were treated with 0.1ug/mL doxycycline for the indicated time as described before (Kwon *et al.*, 2005). The cells were then lysed in RIPA buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 0.25% (wt/vol) Na-deocycholate, 1% (vol/vol) NP-40, 1mM dithiothreitol (DTT), 1mM phenylmethylsulfony fluoride (PMSF)) for the whole cell lysate extraction or in TRIzol reagent (Invitrogen) for RNA extraction (For reverse transcription-polymerase chain reaction (RT-PCR)).

Clinical samples

With informed consent according to the Helsinki Declaration, peripheral blood mononuclear cells (PBMCs) were obtained from five patients with acute-type ATL (nos. 1-5) and two healthy volunteers (nos. 1 and 2). The diagnosis of ATL was established hematologically, and monoclonal HTLV-I provirus integration into the genome was confirmed by Southern blot hybridization in all cases. Subtypes of ATL were defined as described before. (Shimoyama, 1991; Tomita *et al.*, 2007; Uozumi, 2010) Mononuclear cells were isolated from heparinized venous blood samples by Ficoll-Paque gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). Each patient sample contained more than 90% leukemic cells at the time of analysis. All samples were collected at the time of admission to hospital before the patients started chemotherapy. The normal PBMC control nos. 3 and 4 were purchased from the Biologic Specialty Corporation (Colmar, PA).

Immunoblotting (IB)

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer) [50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfony fluoride (PMSF)] supplemented with a protease inhibitor cocktail, followed by IB assays according to the standard protocol.

Quantitative PCR analysis

Total RNA was prepared with TRIZOL reagent and cDNA was generated with SuperScript II reverse transcriptase (Invitrogen), followed by normal RT-PCR or real-time PCR assays as described before (Qing *et al.*, 2005b, 2007). Primer pairs for real-time PCR were as

follows: human PDLIM2, forward 5'-GCCCATCATGGTGACTAAGG, human PDLIM2, reverse 5'-ATGGCCACGATTATGTCTCC; human beta-actin, forward 5'-ATCAAGATCATTGCTCCTCCT, human beta-actin, reverse 5'-GAGAGCGAGGCCAGGATGGA; GAPDH, forward 5'-GCAAATTCCATGGCACCGT, GAPDH, reverse 5'-TCGCCCCACTTGATTTTGG; human DNMT1, forward 5'-GGTTCTTCCTCCTGGAGAATGTC, human DNMT1, reverse 5'-GGGCCACGCCGTACTG; human DNMT3a, forward 5'-GCCTCAATGTTACCCTGGAA, human DNMT3a, reverse 5'-CAGCAGATGGTGCAGTAGGA; human DNMT3b, forward 5'-CCCATTCGAGTCCTGTCATT, human DNMT3b, reverse 5'-GGTTCCAACAGCAATGGACT.

4.4 RESULTS

4.4.1 PDLIM2 is repressed in HTLV-I-transformed T cells and primary ATL samples

4.4.1.1 Both the mRNA and protein levels of PDLIM2 are suppressed in HTLV-I-transformed T cells.

To investigate the mechanism by which Tax escapes from PLDLIM2-mediated suppression for cell transformation and the ultimate oncogenesis in about 5% of HTLV-I carriers with a 40-60-year latency, we examined the expression levels of PDLIM2 in HTLV-I-transformed T cells. In agreement with previous studies describing a high expression of PDLIM2 in various haematopoietic cells, we found relatively abundant PDLIM2 proteins in Jurkat T cells,

an HTLV-I-negative T cell line (Figure 30). Remarkably, PDLIM2 proteins levels were significantly decreased in all of the HTLV-I-transformed T cell lines we examined as well as in 293 and Hela cells, although to the different extents (Figure 30).

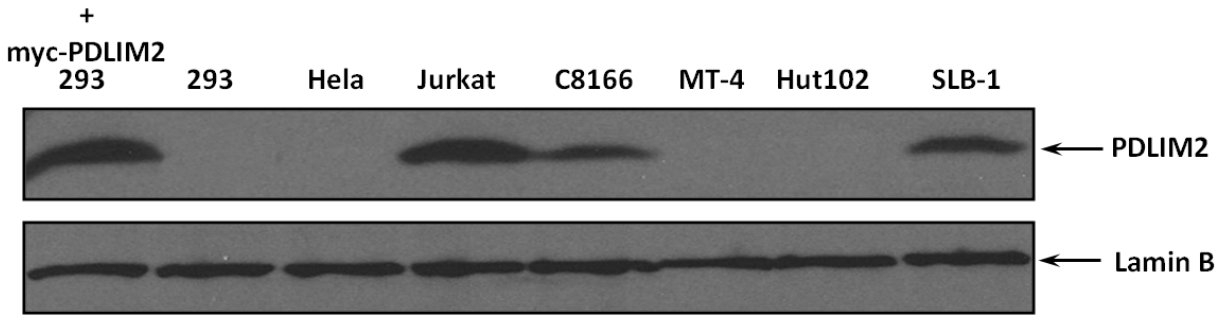

Figure 30. PDLIM2 is repressed in HTLV-I-transformed T cells as well as 293 and Hela cells.

Protein expression of PDLIM2 was measured in the indicated cells by IB using hPDLIM2 antibody. Over-expressed myc-PDLIM2 in 293 cells was used as the positive control.

To examine the mechanism by which PDLIM2 is down-regulated, we performed a CHX-chase assay to check whether it occurs at the protein level. Although PDLIM2 significantly reduces Tax stability, the protein stability of PDLIM2 itself was not affected by Tax or HTLV-I (Figure 18A, Figure 25, Figure 26), excluding that possibility. We then examined the mRNA levels of PDLIM2 in HTLV-I-transformed T cells using real-time PCR. We found that all HTLV-I-transformed T cell lines to maintain low levels of PDLIM2 mRNA compared to Jurkat cells, albeit to different extents, consistent with their protein levels of PDLIM2 (Table 2). The positive correlation between PDLIM2 mRNA and protein levels suggested that PDLIM2 repression occurred at the transcriptional level. Given the role of PDLIM2 expression in prevention of Tax-mediated oncogenesis, these results also suggested that down-regulation of

PDLIM2 transcription is one important mechanism for HTLV-I-mediated tumorigenesis and contribute to HTLV-I infection.

Table 2. Relative mRNA levels of PDLIM2 in HTLV-I-transformed T cells.

Cell line	Jurkat	C8166	MT-4	Hut-102	SLB-1	293	Hela
Percentile	100	54.5 ± 13.6	1.2 ± 0.1	3.3 ± 0.4	55.5 ± 14.6	0.7 ± 0.3	0.3 ± 0.1

4.4.1.2 HTLV-I represses PDLIM2 mRNA level in primary ATL samples.

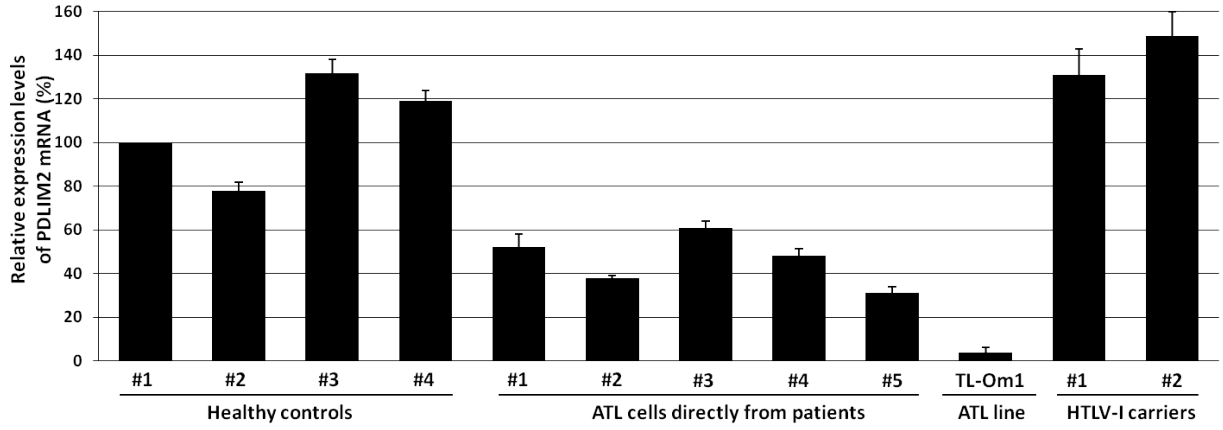

Figure 31. HTLV-I represses PDLIM2 expression in primary ATL samples.

The relative mRNA levels of PDLIM2 in PBMCs directly from ATL patients and HTLV-I carriers and the established ATL cell line TL-Om1 were analyzed by real-time PCR and normalized according to GAPDH mRNA level and represented as percentile in cells from healthy control no. 1 (set as 100).

To further validate the results under physiological conditions, we examined the mRNA levels of PDLIM2 in primary ATL cells as well as viral infected cells from HTLV-I carriers. Indeed, the expression levels of PDLIM2 were much lower in primary ATL cells directly from patients or established ATL cell line TL-Om1 compared to the healthy control cells (Figure 31). Importantly, two HTLV-I carriers showed relatively high levels of PDLIM2 comparable to the

healthy controls (Figure 31). These results suggested that PDLIM2 repression might be an HTLV-I pathophysiological event associated with HTLV-I-mediated cell transformation and oncogenesis. Moreover, such repression may be associated with the progression of ATL and provide an explanation for the long latency of ATL, that ATL generation needs to overcome the high level of PDLIM2.

4.4.2 DNA methylation contributes to HTLV-I-mediated repression of PDLIM2.

4.4.2.1 DNMT1 and DNMT3b, but not DNMT3a, are highly expressed in HTLV-I-transformed T cells.

As described above, HTLV-I-mediated repression of PDLIM2 might be at the transcriptional level. DNA methylation is a well-known mechanism responsible for the repression of a large group of tumor suppressor genes acting independently of genetic alterations. The methylation of mammalian genomic DNA is mediated by three DNA methyltransferases including DNMT1, DNMT3a, and DNMT3b. To identify the DNMTs involved in HTLV-I–mediated PDLIM2 repression, we examined their mRNA levels in various HTLV-I-transformed T cell and ATL cell lines including C8166, MT-4, SLB-1, Hut102, and TL-Om1. As shown in Figure 32, DNMT1 and DNMT3b were highly expressed in all these HTLV-I-transformed T cell or ATL cell lines compared to normal PBMC cells, although to different extents. Conversely, DNMT3a was only slightly up-regulated in certain cell lines (SLB-1 and Hut102) but decreased in others (Figure 32). Thus, it seemed that DNMT1 and DNMT3b but not DNMT3a may be involved in HTLV-I-mediated repression of PDLIM2. Indeed, DNMT1 and DNMT3b usually

cooperate together by forming heterodimers *in vivo*, and inactivate tumor suppressor genes in cancer cells but not in non-malignant cells (Luczak and Jagodzinski, 2006).

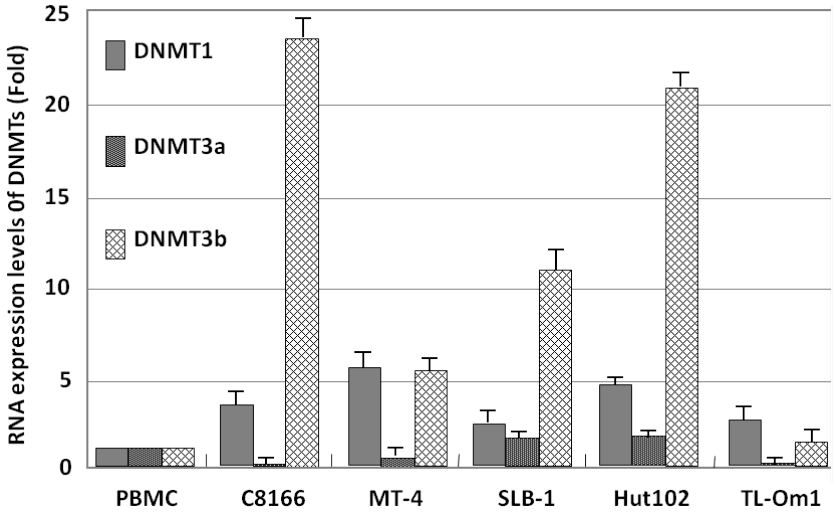

Figure 32. DNMT1 and DNMT3b are highly expressed in HTLV-I-transformed T cells.

RNA expression levels of DNMT1, DNMT3a, and DNMT3b were analyzed in the indicated cell lines by real-time PCR. RNA levels of individual DNMTs in HTLV-I–transformed T cells or ATL cells were normalized according to β-actin mRNA level and represented as fold induction in mRNA abundance relative to that in normal PBMCs (set as 1). Data presented are the mean ± SD (n =3).

Furthermore, the results also implied that the up-regulation of DNMT1 and DNMT3b in HTLV-I-transformed T cells is independent of Tax expression. Because whereas C8166, MT-4, SLB-1, and Hut102 cells still express Tax, the ATL cell line TL-Om1 already loses Tax expression. This is further discussed below.

4.4.2.2 Inhibition of DNMTs results in re-expression of PDLIM2 in HTLV-I-transformed T cells.

According to our results, the repressed PDLIM2 might be result from the over activation of DNMTs and hyper-methylation of PDLIM2 genomic DNA. Notably, Nucleoside analog 5-aza-dC, which is an anti-tumor drug under clinical trial phase III, irreversibly inhibits all three DNMTs. It inhibits DNMTs by forming stable complexes between incorporated 5-aza-dC and the DNMTs, subsequently mimicking the intermediate state. Interestingly, treatment of this DNMT inhibitor 5-aza-dC significantly increased RNA expression of PDLIM2 in T cell lines either *in vitro* transformed by HTLV-I or established from ATL patients (Figure 33). As the control, the treatment of 5-aza-dC led to a light decrease of PDLIM2 in Jurkat cells. These results indicated the demethylation by inhibition of DNMTs is associated with PDLIM2 reactivation. In other words, HTLV-I–mediated repression of PDLIM2 involves DNA methylation.

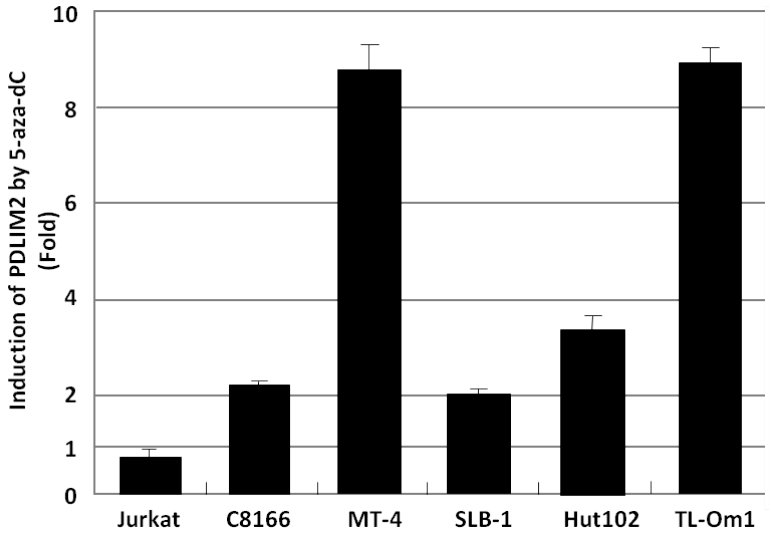

Figure 33: Inhibition of DNMTs reactivates expression of PDLIM2 mRNA.

The indicated cells were treated with 5-aza-dC for 48 hours following by real-time PCR to detect PDLIM2. PDLIM2 mRNA were normalized according to β-actin mRNA level and represented as fold induction in mRNA abundance relative to that in the mock-treated sample of each request. Data presented are the mean ± SD (n = 3).

4.4.2.3 Inhibition of DNMTs induces the death of HTLV-I-transformed T cells in a dose-dependent manner.

Importantly, the treatment of 5-aza-dC resulted in the death of HTLV-I–transformed T cells in a dose-dependent manner (Figure 34). Although 5-aza-dC is unable to further increase PDLIM2 expression in Jurkat cells, it could also induce death of the leukemic cells in a dose-dependent manner, while normal PBMCs were largely resistant to 5-aza-dC–induced cell death. These results suggest that 5-aza-dC induced cancer cell death may involve different target genes. Nevertheless, it is consistent with the fact that 5-aza-dC is toxic to cancer cells but not normal cells. Currently, there is no beneficial treatment for ATL patient save bone marrow transplant given the multidrug resistance of ATL cells observed during chemotherapy. Our results may therefore provide a novel therapeutic target for ATL, although 5-aza-dC targets other than PDLIM2 need to be identified.

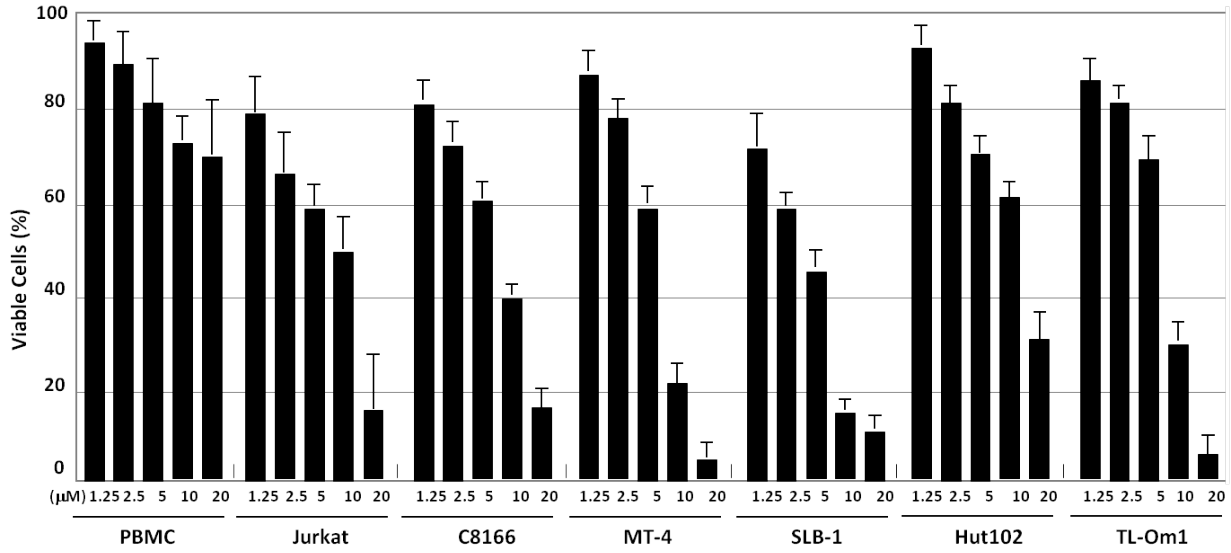

Figure 34. Inhibition of DNMTs induces the death of HTLV-I-transformed T cells.

The indicated cells were treated with increasing dosages of 5-aza-dC for 72 hours followed by Trypan blue counting of dead and viable cells. The presented percentiles of viable cells were normalized to the percent viability of mock-treated cells (set as 100%). Data presented are the mean ± SD (n = 3)

4.4.3 Repression of PDLIM2 by HTLV-I is independent of oncoprotein Tax.

4.4.3.1 Tax expression does not affect PDLIM2 mRNA level.

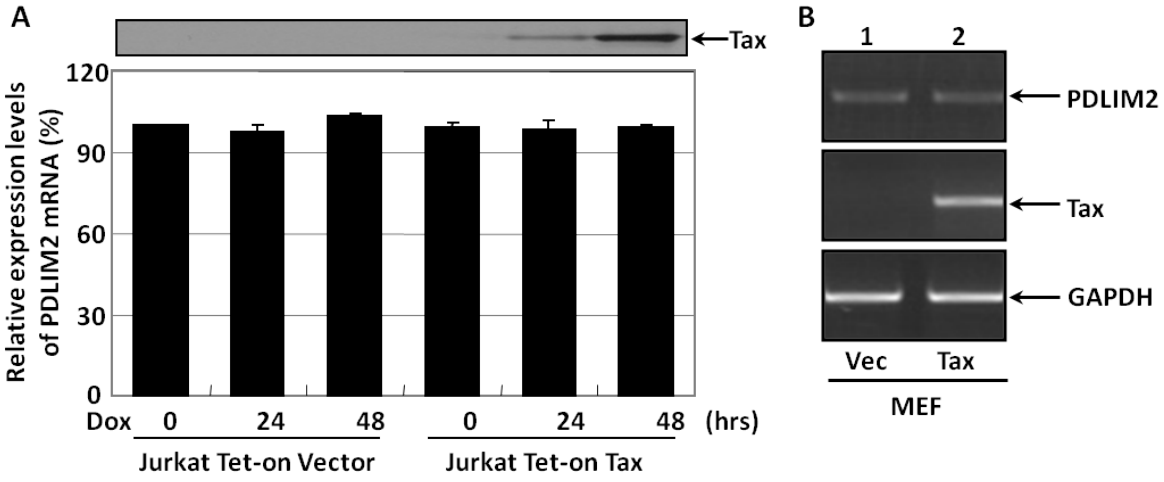

Figure 35. Tax expression does not affect PDLIM2 mRNA level.

(A) Tax-inducible Jurkat cells were mock-treated or treated with Dox for the indicated times followed by real-time PCR. The induction of Tax protein was analyzed by direct IB analysis. (B) MEF cells stably expressing Tax or an empty vector were used for RT-PCR to check expressions of the indicated genes.

The mechanism of PDLIM2 regulation, including that by which PDLIM2 expression is repressed by HTLV-I, remains largely unknown. We initially proposed that HTLV-I Tax oncoprotein is involved, giving its role in gene regulation and cellular transformation. Consistent with our previous studies showing that Jurkat cells, an HTLV-I–negative leukemic T cell line expressed abundant PDLIM2, we found a high expression of PDLIM2 in Tax-inducible Jurkat cells in the absence of Tax (Figure 35A). However, Tax induction had no obvious effect on PDLIM2 expression, indicating that PDLIM2 is relatively stable during the short-term Tax expression (Figure 35A). It should be noted that Jurkat cells died 72 to 96 hours following Tax

induction because of Tax-induced TRAIL and the subsequent TRAIL-mediated apoptosis. These Tax-inducible Jurkat cells cannot be used for long term expression of Tax.

To rule out that the failure of the PDLIM2 repression was not due to insufficient function of transient induced Tax, we compared the PDLIM2 expression levels in MEF cells stably expressing Tax or an empty vector. Noteworthy, Tax-expressing MEF cells acquired the ability to form foci in soft agar as well as tumors in mice. However, as shown in Figure 36B, long-term expression of Tax in these transformed cells still failed to inhibit PDLIM2 expression. In agreement with this, PDLIM2 expression is also repressed ATL cell line TL-Om1 (Figure 31), which already lost Tax expression. Altogether, our experiments suggest that HTLV-I repressed PDLIM2 expression is independent of Tax.

4.4.3.2 Inducible expression of Tax in Jurkat cells does not affect DNMTs' mRNA level.

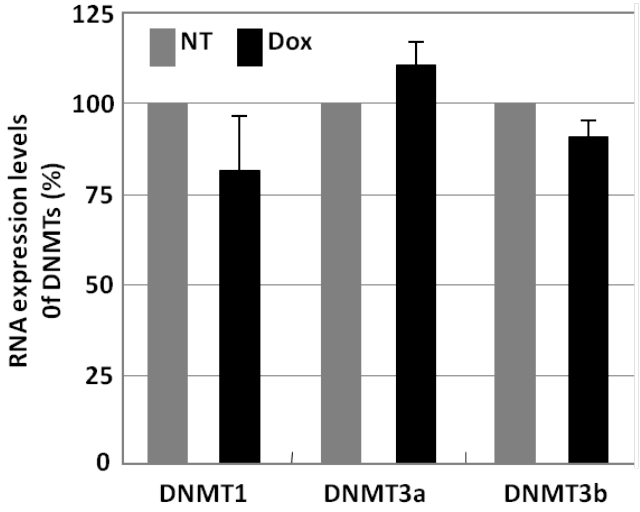

Figure 36. Inducible expression of Tax in Jurkat cells does not affect DNMTs' mRNA.

Tax-inducible Jurkat cells were mock-treated or treated with Dox for 36 hours followed by real-time PCR to check the relative RNA expression levels of DNMT1, DNMT3a, and DNMT3b. RNA levels of individual DNMTs in Dox-treated cells were normalized according to β-actin mRNA level and represented as percentile of that in the mock-treated (NT) cells (set as 100). Data presented are the mean ± SD (n = 3).

Considering the link between DNMTs and PDLIM2, we set t0 examine whether Tax induction could affect the expression of different DNMTs in Jurkat cells. As shown in Figure 36, Tax induction had no obvious effect on the expression of all three DNMTs. Taken them together, the results suggest that Tax was not involved in the up-regulation of DNMT1 and DNMT3b in HTLV-I–transformed T cells and ATL cells, further supporting the notion that PDLIM2 repression by HTLV-I is independent of HTLV-I Tax.

4.5 DISCUSSION

Our mechanistic studies suggest that HTLV-I-mediated PDLIM2 repression involves DNA methylation, which might be mediated by DNMT1 and DNMT3b. We have shown that the DNMT inhibitor 5-aza-dC could significantly reactivate transcription of PDLIM2 in both HTLV-I-transformed T cells and ATL cells. In addition, DNMT1 and DNMT3b, but not DNMT3a, were significantly and consistently up-regulated in these malignant T cells. In accordance with the reactivation of PDLIM2, 5-aza-dC could efficiently induce death of HTLV-I-transformed T cells and ATL cells. This is particularly interesting because HTLV-I-transformed cells are highly resistant to the induction of apoptosis and there is still no beneficial treatment other than allogeneic hematopoietic stem cell transplantation for this acute and fatal disease. Thus, this finding may provide novel therapeutic approaches for ATL therapy. Clearly, the effect of 5-aza-dC could not attribute only to the reactivation of PDLIM2. Other undefined targets of this antitumor drug also may play more important roles in the death of HTLV-I-transformed T cells.

Although Tax plays a critical role in the initial stage of HTLV-I-mediated leukemogenesis, our studies suggested that HTLV-I represses PDLIM2 expression independent

of Tax. First, PDLIM2 was repressed in Tax-negative ATL cells in addition to Tax-positive HTLV-I–transformed T cells. Second, Tax was dispensable for the specific up-regulation of DNMT1 and DNMT3b in HTLV-I-transformed T cells, although DNA methylation played an important role in the PDLIM2 repression in these malignant cells. Most importantly, neither short term induction nor long-term stable expression of Tax could repress PDLIM2 transcription.

In summary, our studies indicate that HTLV-I-mediated repression of PDLIM2 occurred under pathophysiological conditions, contributing to the long latency of HTLV-I infection, and that the PDLIM2 repression involves DNA methylation possibly through specific up-regulation of DNMT1 and DNMT3b. Our studies also excluded the role of Tax in the PDLIM2 repression. Given the death of HTLV-I-transformed cells triggered by the antitumor drug 5-aza-dC, these studies not only help understand regulation of PDLIM2, leukemogenicity, long latency and cancer health disparities of HTLV-I, but also suggest a direct therapeutic strategy for ATL.

5.0 IMPLICATION FOR P65-INDEPENDENT NF-KB ACTIVATION IN REGULATION OF TAX BY PDLIM2

Research in this Chapter is still under process and preparing for submission with authors Pengrong Yan and Gutian Xiao.

*Data showed in this section is preliminary and needs further validations.

5.1 SUMMARY

HTLV-I Tax exerts its oncogenic role through deregulation of cellular transcription factors that are critical for cell growth and division, mainly through NF-κB signaling pathway. PDLIM2 is an intrinsic negative regulator of HTLV-I Tax and terminator of p65-mediated NF-κB activation. We found that Tax-binding-defective mutant of PDLIM2, which fails to degrade HTLV-I Tax and inhibits Tax-mediated tumor growth, retains the ability to interact with p65, alter p65 subcellular distributions and suppress p65-mediated NF-κB activation. Additionally, the PDLIM2 defective mutant is unable to inhibit p52-mediated NF-κB activation. The result therefore indicates the implication for p65-independent mechanisms in PDLIM2 suppressed NF-κB activation as well as Tax-mediated tumorigenesis.

5.2 INTRODUCTION

As described before, p65, PDLIM2 and Tax are all involved in NF-κB signaling pathway, and either two of them interact with each other and function distinctly. We found them to have more complicated relationships: 1) PDLIM2 is the E3 ligase of nuclear p65, triggering its proteasomal degradation and shutting off p65-mediated NF-κB activation. The LIM domain is required for this process, while the PDZ domain within PDLIM2 might have the intra-nuclear trafficking function for p65 (Tanaka *et al.*, 2007). 2) PDLIM2 promotes the poly-ubiquitination and the subsequent proteasomal degradation of Tax. Additionally, PDLIM2 interacts with Tax via its internal putative α-helix, named Tax-binding motif (TBM). Both the LIM domain and the TBM are required for suppressing Tax mediated cell transformation and tumorigenesis, but the PDZ domain is dispensable for all the events (Table 3, Figure 28, Figure 29). 3) Interaction between p65 and Tax mainly appears in the nucleus and is associated with Tax nuclear bodies, which contribute to the full Tax-mediated NF-κB activation. Moreover, evidence showed that Tax could induce the phosphorylation of p65, which leads to the expression of MDM2 and inactivation of p53 (Jeong *et al.*, 2004; Suzuki *et al.*, 1999b).

Tax intervenes at multiple levels to activate NF-κB. Tax-activated NF-κB has been demonstrated to be crucial for Tax-mediated cell transformation and tumorigenesis (Kwon *et al.*, 2005; Sun and Yamaoka, 2005). p65 is one of the major targets of Tax, and phosphorylation of p65 is required for the Tax-induced NF-κB activation (O'Mahony *et al.*, 2004). Other than p65/p50, the prototypic transactivator in the canonical NF-κB pathway, Tax also activates non-

canonical pathway by IKK activation and subsequently p100 processing to p52. Moreover, the aberrant over-production of p52 is considered a hallmark of HTLV-I infection and transformation in ATL (Xiao *et al.*, 2001a). Despite lacking solid evidence, a p65-independent mechanism might contribute to Tax-mediated NF-κB activation under some conditions. The hypothesis is supported by the observations that NF-κB DNA-binding complexes in Tax-expressing T cells compose of abundant c-Rel, p50 and p52, but little p65 (Xiao *et al.*, 2001a). Importantly, p65 is still indispensable for Tax-mediated cell transformation because Tax failed to transform the p65-deficient MEF *in vitro* (unpublished data). In sum, both p65-dependent and independent NF-κB seem critical for Tax-mediated NF-κB as well as Tax-mediated tumorigenesis.

Former sections of this thesis focused on elucidating the mechanisms by which PDLIM2 regulates of Tax. However, the role of p65 cannot be ignored based on the pivotal roles of both Tax and p65 in NF-κB signaling. As shown in Chapter 3, Tax-binding-defective mutant of PDLIM2 failed to suppress Tax-mediated cell transformation and tumorigenesis. Consistently, the PDLIM2 defective mutant was unable to promote poly-ubiquitination and proteasomal degradation of Tax. Considering the important role of NF-κB signaling in Tax-mediated tumorigenesis, we took advantage of the Tax-binding-defective mutants of PDLIM2 to dissect the role of p65-dependent NF-κB signaling in Tax-mediated oncogenesis.

5.3 MATERIALS AND METHODS

Expression vectors and reagents

Expression vectors encoding Tax, PDLIM2 and PDLIM2 mutants have been described in Chapter 3.The HRP-conjugated HA monoclonal antibody (3F10) was from Roche Molecular Biochemicals. The Sp1, lamin B, Hsp90, and β-actin antibodies as well as the pre-immune IgG were from Santa Cruz Biotechnology. The flag antibody (M2) was purchased from Sigma. The Tax and MYC antibodies were described previously (Qu *et al.*, 2004).

Cell culture and transfection

HEK293 cells and MEF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 2 mM L-glutamine (Invitrogen). 293 were transfected with DEAE-Dextran (Sigma) (Qing and Xiao, 2005; Xiao and Sun, 2000), and MEF cell with Lipofectamine 2000 (Invitrogen).

Luciferase gene reporter assays

Jurkat, 293 and MEF cells were transfected with luciferase reporter and Tax in the presence of increasing amounts of PDLIM2. At 40 hrs post-transfection, luciferase activity was measured as we described before (Xiao *et al.*, 2000).

Subcellular fractionation, immunoblotting (IB) and immunoprecipitation (IP) assays

Cytoplasmic, soluble and insoluble nuclear extracts were prepared using the hypotonic buffer (20 mM HEPES, pH 8.0, 10 mM KCl, 1 mM $MgCl_2$, 0.1% (vol/vol) Triton X-100 and 20% (vol/vol) glycerol), hypertonic buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 20% (vol/vol) glycerol, 0.1% (vol/vol) Triton X-100 and 400 mM NaCl), and insoluble buffer (20 mM Tris, pH

8.0, 150 mM NaCl, 1% (wt/vol) SDS, 1% (vol/vol) NP-40 and 10 mM iodoacetamide), respectively (Tanaka *et al.*, 2007). The purity of the obtained fractions was confirmed by checking Hsp90 (cytoplasm), Sp1 (soluble nuclear fraction), or lamin B (insoluble nuclear fraction). Total nuclear extracts were prepared by simply lysing pellets in insoluble buffer after the cytoplasm was extracted. Whole-cell extracts were prepared by lysing cells in RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 0.25% (wt/vol) Na-deoxycholate, 1% (vol/vol) NP-40, 1 mM DTT). All the lysis buffers were supplemented with 1 mM PMSF and a protease inhibitor cocktail (Roche Molecular Biochemicals). The cells extracts were used for IP and/or IB assays as described before (Xiao *et al.*, 2001a).

5.4 RESULTS

5.4.1 Tax-binding-defective mutant of PDLIM2 retains the capacity to suppress p65-mediated NF-κB activation.

PDLIM2 functions to target p65 for proteasomal degradation and shut off the p65-mediated NF-κB activation. Previous data showed a tight correlation between Tax-binding capacities of PDLIM2 and the suppression of Tax-mediated tumorigenesis by PDLIM2. In order to understand the functions on NF-κB of the PDLIM2 defective mutants in interacting with Tax, we examined the effects of the mutants on NF-κB activation via luciferase gene reporter assays.

In the absence of Tax, the co-expression of the PDLIM2 mutant (Δ243-253) with WT p65 resulted in a dose-dependent suppression of p65-mediated NF-κB activation, suggesting that the PDLIM2 mutant retains the function as a terminator of NF-κB activation (Figure 37A).

Surprisingly, similar dose-dependent suppression of Tax-mediated NF-κB activation by the PDLIM2 mutant was observed in the presence of Tax (Figure 37B). The result indicates that the outcome of PDLIM2 on Tax-mediated NF-κB activation is dependent on the suppression of p65 by PDLIM2, which will be further confirmed in the following. Importantly, Tax also contributes to HTLV-I viral infection by Tax-mediated HTLV-I-LTR activation, which is NF-κB independent. Our results showed that unlike the WT PDLIM2, the Tax-binding-defective mutant was incapable to suppress Tax-mediated LTR activation (Figure 37C).

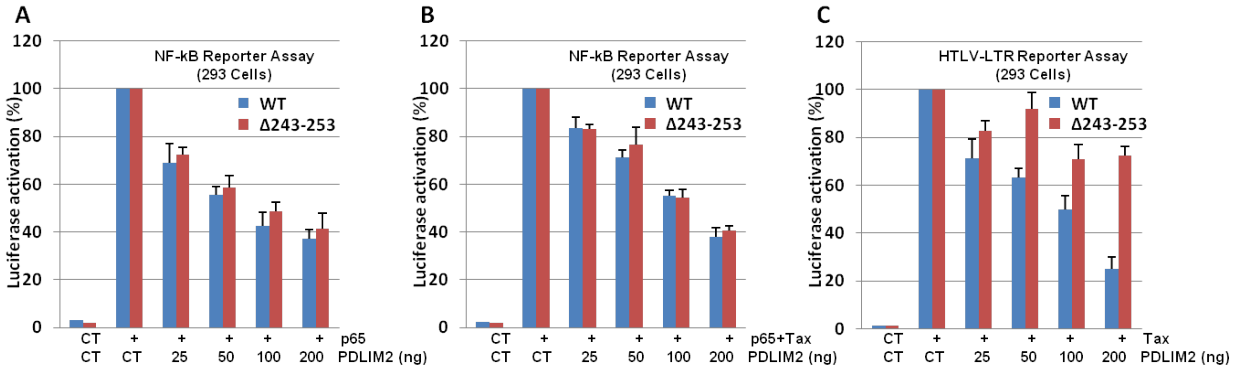

Figure 37. Tax-binding-defective mutant of PDLIM2 retains the capacity to suppress p65-mediated NF-κB activation.

(A) 293 cells were transfected with p65 and κB driven luciferase reporter in the presence of increasing amounts of WT and mutant of PDLIM2, followed by the measurement of luciferase activity. (B) 293 cells were transfected with p65, Tax and κB driven luciferase reporter in the presence of increasing amounts of WT and mutant of PDLIM2, followed by the measurement of luciferase activity. (C) 293 cells were transfected with Tax and HTLV-LTR driven luciferase reporter in the presence of increasing amounts of WT and mutant of PDLIM2, followed by the measurement of luciferase activity.

Since the suppression of p65-mediated NF-κB activation by PDLIM2 is associated with the binding ability to p65 and altering p65 subcellular distribution, we performed co-IP and cell fraction assays to confirm the functions of the Tax-binding-defective mutant of PDLIM2.

5.4.2 Tax-binding-defective mutant of PDLIM2 still interacts with p65.

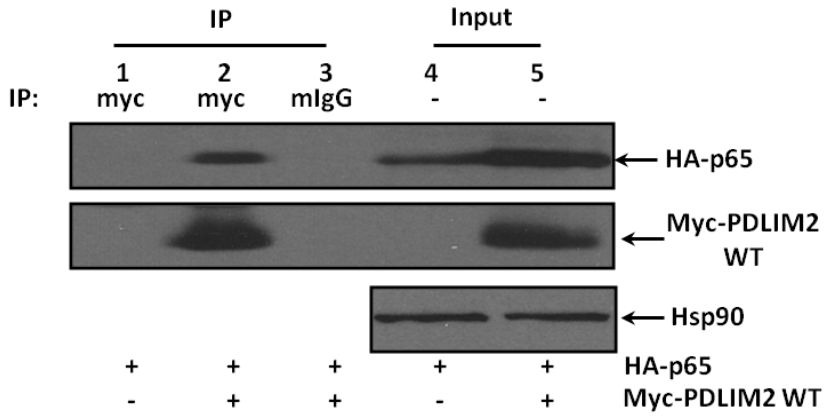

Figure 38. PDLIM2 interacts with nuclear p65.

293 cells were transfected with HA-p65 in the presence or absence of WT Myc-PDLIM2, following by IP using myc antibody and IgG, and IB using Tax antibody. The expression of input Tax and myc-PDLIM2 were analyzed by direct IB. Hsp90 was used as the loading control.

Since PDLIM2 functions to trigger nuclear p65 for degradation, we utilized the nuclear fractions to address the interaction between PDLIM2 and p65. In agreement with previous studies, p65 physically interacted with WT PDLIM2 in the co-IP assay (Figure 38). After using the Tax-binding-defective mutants of PDLIM2, the interactions between p65 and PDLIM2 mutants were still kept at comparable levels in the absence of Tax (Figure 39, lane 2 vs lanes 3-6), suggesting that the binding of PDLIM2-Tax and PDLIM2-p65 are specific to each other (Figure 21A, Figure 22, Figure 39). In other words, different sequences within PDLIM2 are responsible for these two interactions.

Interestingly, in the presence of Tax, despite the ability of all Tax-binding-defective mutants to interact with p65, the WT PDLIM2 protein showed a slightly decreased binding capacity compared to the binding-defective mutant (Figure 39, lane 8 vs lanes 9-12). A similar

result was observed and confirmed when using a PDLIM2 protein bearing a different epitope tag (Figure 40). This evidence suggested the possibility that Tax presence may compete with p65 in occupying PDLIM2. Considering that under our experimental condition, the over-expressed p65 was more abundant than Tax in the nucleus, the results suggested that PDLIM2 might prefer to interact with Tax.

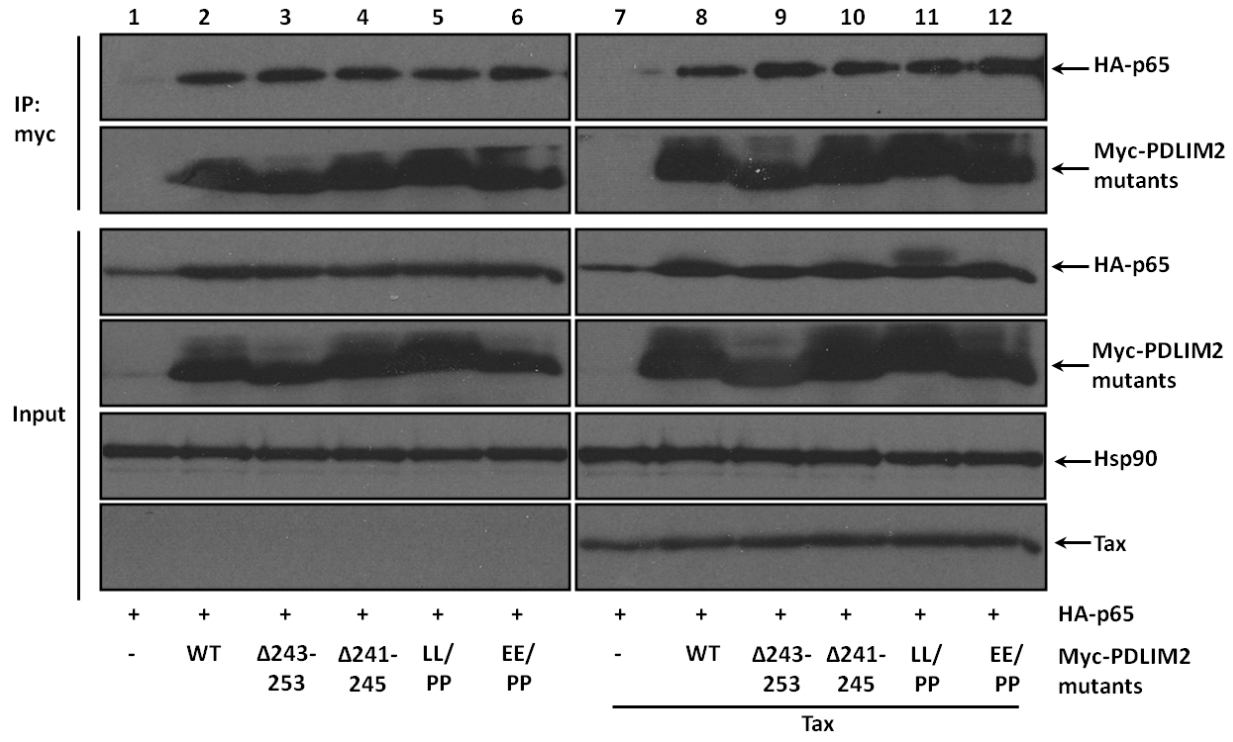

Figure 39. Tax-binding-defective mutant of PDLIM2 interacts with p65 in the absence and presence of Tax. 293 cells were transfected with HA-p65 in the presence of the indicated PDLIM2 mutants, following by IP using myc antibody, and IB using HA-HRP antibody. The expression of input HA-p65, myc-PDLIM2 and Tax were analyzed by direct IB. Hsp90 was used as the loading control for inputs.

To further confirm whether Tax competes with p65 in occupying PDLIM2, and whether there was a large p65-PDLIM2-Tax scaffold protein complex in the cells, we precleared the cell lysates by using anti-Tax-agarose beads, and performed the second co-IP assay to measure the

binding capacity between PDLIM2 and p65. Consistently, presence of Tax leads to a reduced binding capacity to p65 of PDLIM2 (Figure 41, lanes 7&8 vs lanes 3&4). Of note, upon depletion of Tax containing protein complexes, the PDLIM2-p65 interaction rarely changed compared to the untreated samples (Figure 41, lane 9 vs lane 7). This suggested that Tax and p65 preferentially formed separate complexes with PDLIM2, rather than one large p65-PDLIM2-Tax complex. However, the data cannot exclude the possibility that p65-PDLIM2-Tax might be a dynamic intermediate product.

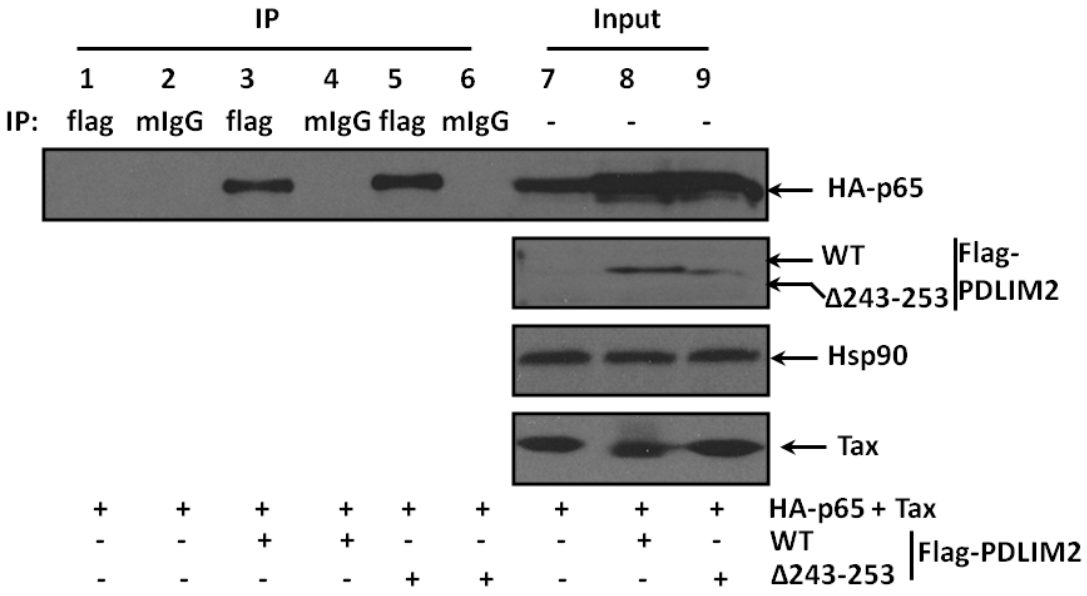

Figure 40. Tax-binding-defective mutant of PDLIM2 still binds to p65 in the presence of Tax.

293 cells were transfected with HA-p65 in the presence of WT and the PDLIM2 mutant Δ243-253, following by IP using myc antibody, and IB using HA-HRP antibody. The expression of input HA-p65, myc-PDLIM2 and Tax were analyzed by direct IB. Hsp90 was used as the loading control.

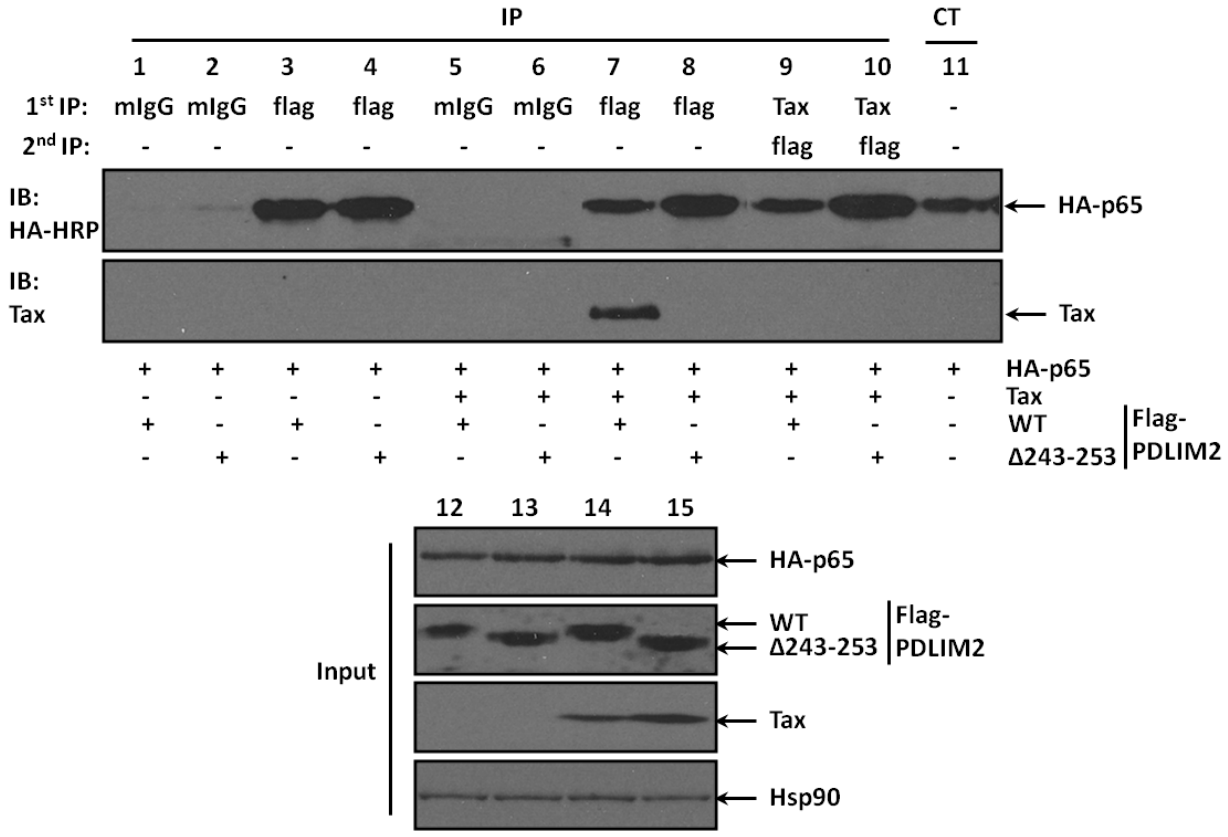

Figure 41. Depletion of Tax-containing complex does not affect PDLIM2-p65 interaction.

293 cells were transfected with HA-p65 and Tax in the presence of WT and the PDLIM2 mutant Δ243-253, following by IP using myc antibody, and IB using HA-HRP antibody. For the lanes 9 & 10, 1st IP was performed by using agarose bead with Tax antibody. After incubation for 3hrs at 4°C, the supernatant was collected by brief centrifuge and subjected to 2nd IP using flag antibody. The expression of input HA-p65, myc-PDLIM2 and Tax were analyzed by direct IB. Hsp90 was blotted as a loading control.

5.4.3 Tax-binding-defective mutant of PDLIM2 retains the abilities to alter p65 subcellular distribution.

PDLIM2 alters the subcellular distribution of p65. The over-expression of WT PDLIM2 results in a significant decrease of p65 in the cytoplasm and nucleoplasm, and elevated p65 levels in the insoluble nuclear fraction, which is in accordance with PDLIM2-mediated

proteasomal degradation of p65 in the nuclear PML bodies. We thus performed the subcellular fraction assays using 293 cells to address the issue of whether those PDLIM2 mutants with Tax-binding defect could alter the subcellular distribution of p65.

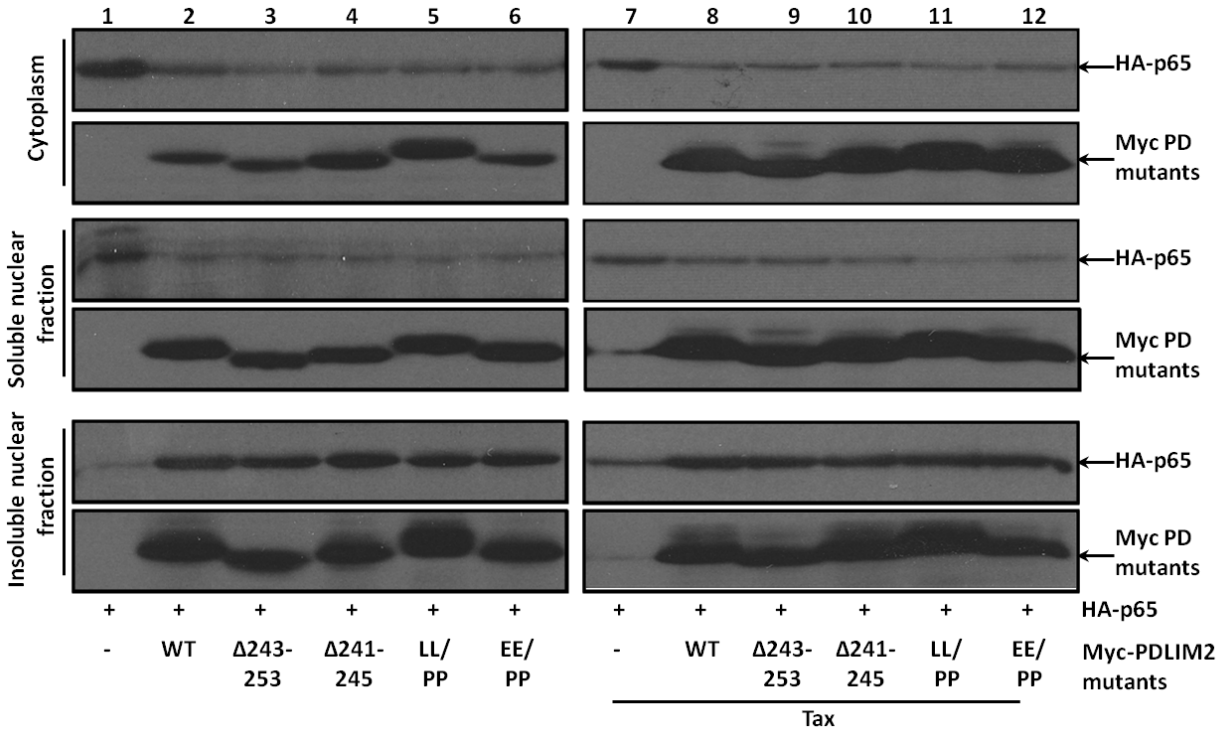

Figure 42. Tax-binding-defective mutant of PDLIM2 retains the ability to alter p65 subcellular distribution. 293 cells were transfected with p65 alone or together with the indicated PDLIM2 mutants in the absence (Lane 1-6) and presence of Tax (Lane 7-12), followed by cell fractions and IB to detect expression levels of the indicated proteins. The subcellular distribution of Tax is consistent with Figure 28 and not shown here.

Consistent with the over-expressed p65, PDLIM2 expression led to a dramatic reduction of p65 in the cytoplasmic and nucleoplasmic fractions, as well as an increased p65 level in the insoluble nuclear fraction. Meanwhile, the disruption of Tax-binding did not present crucial differences between the WT and mutants of PDLIM2 in altering p65 distribution (Figure 42, lanes 1-6). Despite being affected at the different extents, similar results were obtained in the

presence of Tax, indicating that all those PDLIM2 mutants retained the ability to alter p65 subcellular distributions just as the WT protein (Figure 42, lane 7-12). This is in agreement with the fact that they kept the ability to interact with p65 and suppress p65 mediated NF-κB signaling.

Of note, previous studies showed that such function of PDLIM2 depended on both the N-terminal PDZ domain and the C-terminal LIM domain: PDZ domain was responsible for the intra-nuclear trafficking of p65, while LIM domain functions as ubiquitin E3 ligase of p65 (Tanaka *et al.*, 2007). The above results supported the fact because all the tested Tax-binding-defective mutants of PDLIM2 still maintained the full sequences of both PDZ and LIM domains.

5.4.4 Suppression of Tax-mediated NF-κB by PDLIM2 involves p65-independent but p52-dependent mechanisms.

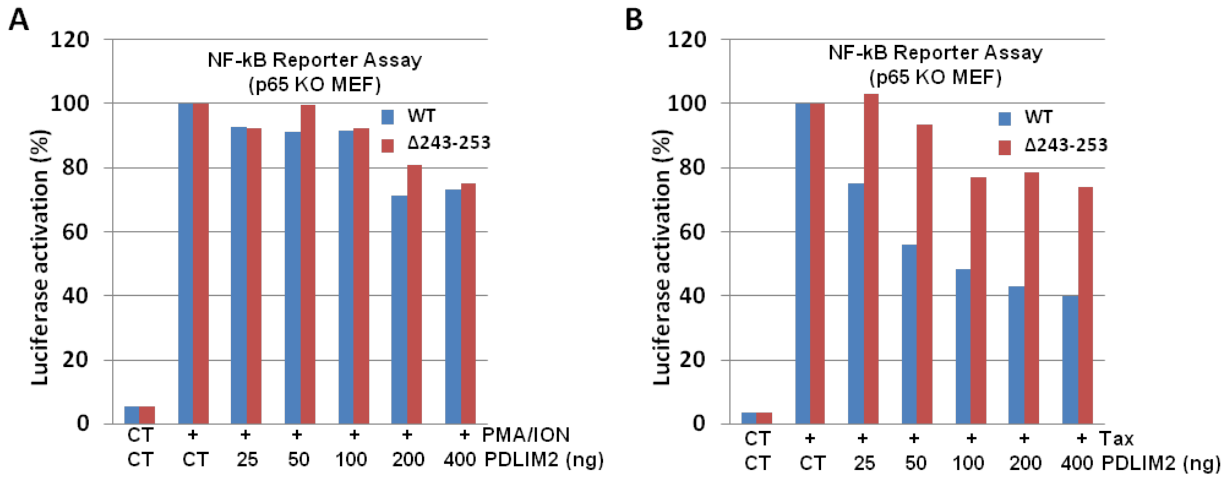

Figure 43. Tax regulation by PDLIM2 involves p65-independent mechanism.

(A, B) The p65 KO MEF cells were transfected with Tax (or subject to PMA/ION treatment) and κB driven luciferase reporter in the presence of increasing amounts of PDLIM2, followed by the measurement of luciferase activity.

Based on the former results, the WT and Tax-binding-defective PDLIM2 mutants function similarly on p65-mediated signaling. However, they showed different effects on suppressing Tax-mediated cell transformation and tumorigenesis. Taking advantage of p65-deficient MEF cells, we dissected the p65-dependent NF-κB signaling in Tax-mediated NF-κB activation. As shown in Figure 45B, the phorbol-12-myristate-13-acetate/Ionomycin (PMA/ION)-induced NF-κB activation in the p65-deficient MEF cells was insensitive to the over-expression of either WT or mutant of PDLIM2, confirming the crucial role of p65 in PDLIM2 suppressed NF-κB. However, the WT PDLIM2, but not the mutant, could suppress Tax-induced NF-κB activation (Figure 43B).

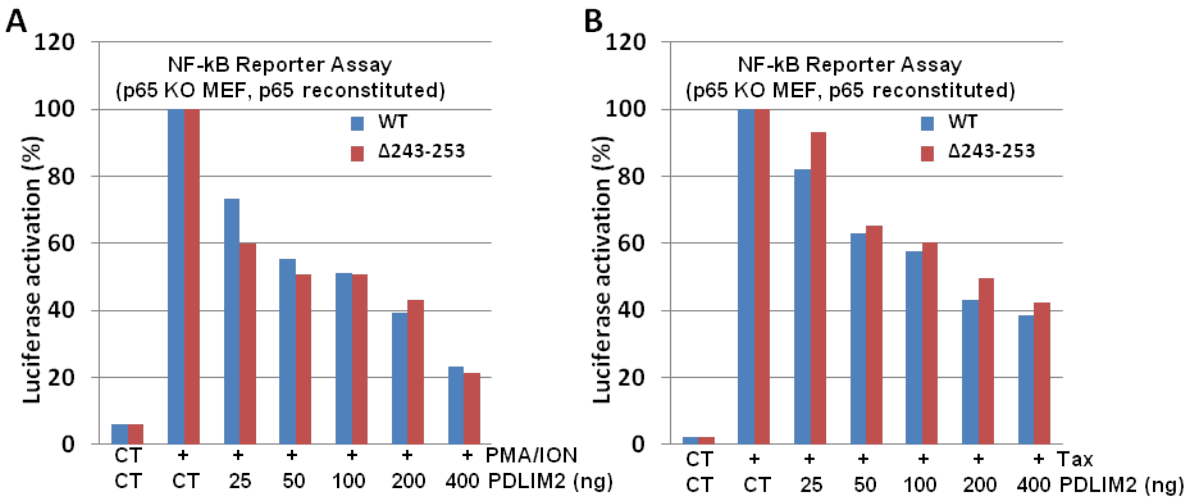

Figure 44. Reconstitution of p65 in p65 KO MEF restores the suppression function of PDLIM2 on NF-κB.

(A, B) The p65 KO MEF cells with reconstituted WT p65 were transfected with Tax (or subject to PMA/ION treatment) and κB driven luciferase reporter in the presence of increasing amounts of PDLIM2, followed by the measurement of luciferase activity.

In addition, we reconstituted the p65 expression in those p65-deficient MEF cells and performed similar assays. Interestingly, suppression of NF-κB by both WT and mutant PDLIM2

was observed (Figure 44). The results did not only indicate that both WT and the Tax-binding-defective mutant of PDLIM2 mainly suppressed NF-κB by targeting p65, but also suggested that other NF-κB members were involved in the suppression of Tax-mediated NF-κB activation. In other words, suppression of Tax-mediated NF-κB by PDLIM2 involved p65-independent mechanisms.

Because the difference between the WT and mutant of PDLIM2 resided on the binding capacity to Tax and triggering Tax degradation, we assumed that PDLIM2 functioned to modulate the Tax protein level in order to indirectly suppress Tax-mediated NF-κB in the absence of p65.

Based on the importance of p52 in Tax-mediated NF-κB, we tested its involvement in such p65-independent mechanisms. In accordance with previous studies on p52, over-expressed p52 appeared to be a weak inducer of NF-κB transcriptional activation in a unique manner (Figure 45A). By using a p52 dosage with maximal NF-κB activation in p65-deficient MEF cells, we showed that the p52-mediated NF-κB activations was relatively resistant to both WT and mutant PDLIM2 in the absence of Tax (Figure 45B), while only the PDLIM2 WT exhibits its suppressive effects in the presence of Tax (Figure 45C). These results suggest that other than p65, p52 is another NF-κB member involved in PDLIM2-mediated suppression of Tax-induced NF-κB, although whether p52 is a client of PDLIM2 remains to be determined. Additionally, in agreement with the lack of interaction between PDLIM2 and p50 (Tanaka *et al.*, 2007), neither WT PDLIM2 nor Tax-binding-defective mutant suppressed p50-mediated NF-κB activation in p65-deficient cells, ruling out the involvement of p50 (Figure 45D).

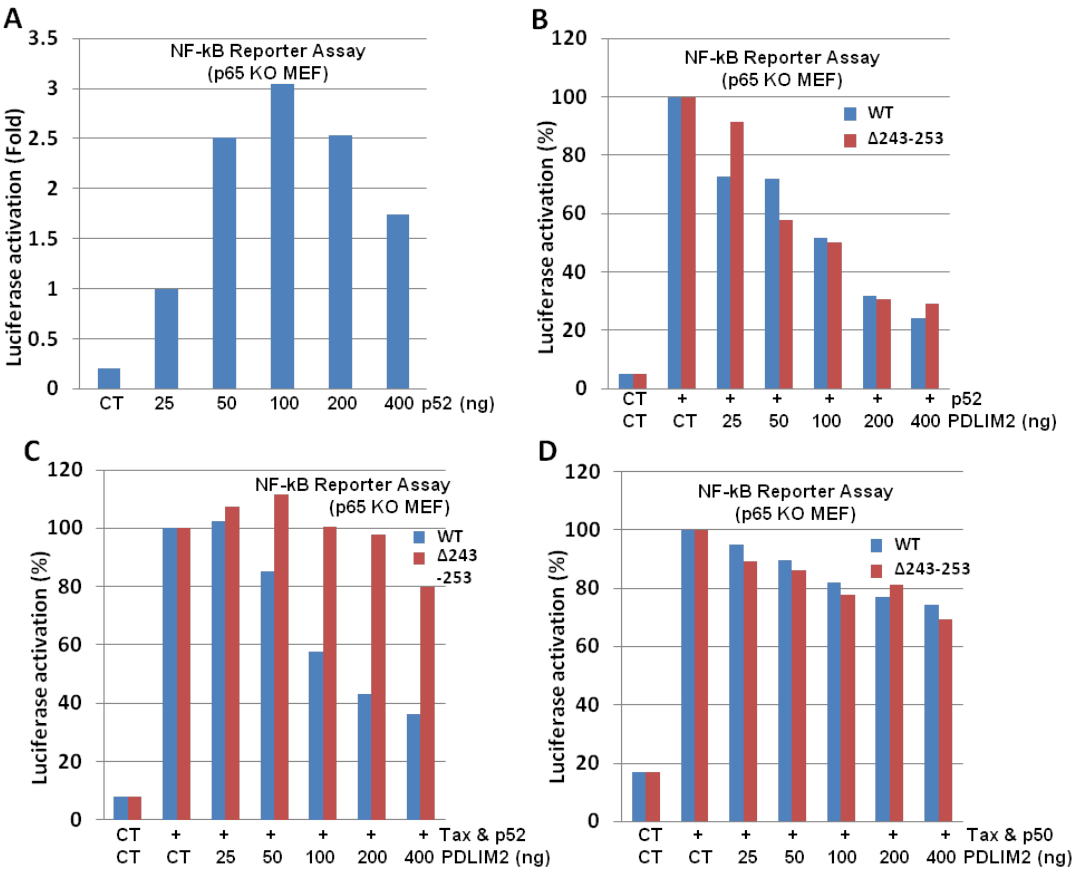

Figure 45. PDLIM2 regulates p52-mediated NF-κB transcriptional activation.

(A) P65 KO MEF cells were transfected with κB driven luciferase reporter in the presence of increasing amounts of p52, followed by the measurement of luciferase activity. (B, C, D) P65 KO MEF cells were transfected with p52 (Tax & p52 or Tax & p50) and κB driven luciferase reporter in the presence of increasing amounts of PDLIM2, followed by the measurement of luciferase activity.

5.5 DISCUSSION

In this Chapter, our preliminary results dissected p65-dependent NF-κB activation in regulation of Tax-mediated NF-κB and oncogenesis by PDLIM2. Taken advantage of the Tax-binding-defective mutant of PDLIM2, which is unable to interact with Tax and trigger its

proteasomal degradation as well as Tax-mediated cell transformation and tumor growth (Chapter 3), we found that those defective PDLIM2 keeps binding to p65, altering p65 subcellular distributions and suppressing p65-dependent NF-κB activation. Additionally, unlike the WT PDLIM2, the Tax-binding-defective mutant fails to suppress p52-mediated p65-independent NF-κB activation, suggesting the involvement of p65-independent mechanisms in Tax regulation. Importantly, the ability of PDLIM2 to bind to Tax is in accordance with suppressive Tax-mediated cell transformation and tumor growth (Figure 28, 29). Our results therefore implicate the pertinent role of p65-independent NF-κB in Tax-mediated oncogenesis.

Notably, p65-mediated NF-κB is still required for Tax-mediated cell transformation since Tax fails to transform the p65-deficient cells *in vitro* (unpublished data). Both p65-dependent and p65-independent (especially p52-dependent) NF-κB seem critical for Tax-mediated tumorigenesis. Detailed discussion and proposed signaling model will be presented in Overall Discussion (Figure 47). In brief, PDLIM2 suppresses Tax mediated NF-κB signaling and oncogenesis at multiple levels. The suppressive function of PDLIM2 involves p65-dependent and p65-independent mechanisms in NF-κB regulation. For the p65-dependent NF-κB activation, PDLIM2 directly targets the proteasomal degradation of p65; while for the Tax-mediated p65-independent NF-κB activation, PDLIM2 directly targets and degrades Tax, resulting in the suppressed Tax protein levels. Similarly to NF-κB regulation, both p65-dependent and p65-independent mechanism are required for Tax-mediated oncogenesis. However, the suppressive outcome of Tax-mediated tumor growth by PDLIM2 may be determined by the regulation of Tax protein as well as p65-independent signaling, rather by a p65-dependent mechanism. Our studies dissected p65-dependent NF-κB signaling in HTLV-I Tax-mediated signaling as well as Tax-mediated oncogenesis.

6.0 OVERALL DISCUSSION

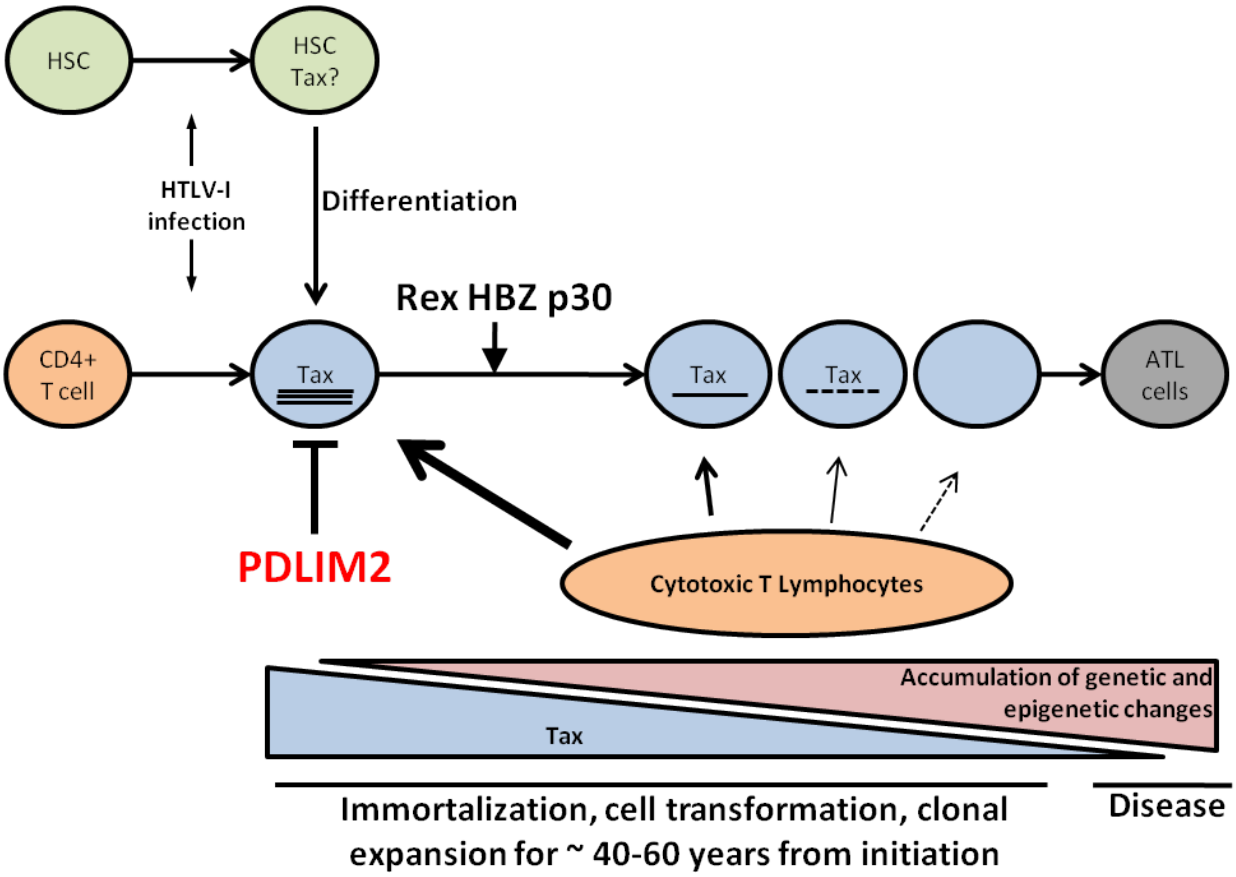

Figure 46. PDLIM2 in HTLV-I infection and ATL development.

During the past thirty years, extensive studies have been performed to address the regulatory mechanisms, by which Tax deregulates cellular signaling to facilitate HTLV-I viral replication and initiate malignant transformation leading to the development of ATL. However, how Tax protein itself is regulated is largely unknown. Although it is accepted that the transient

HTLV-I transcription and expression of the oncoprotein Tax contributes to ATL progression by escaping host immune responses, the benefit of Tax repression for the host is still under investigation. In this thesis we characterized PDLIM2 as a novel intrinsic negative regulator of HTLV-I Tax. PDLIM2 not only determines Tax expression levels, but is also able to prevent HTLV-I/Tax-mediated cell transformation and oncogenesis.

As a matter of fact, PDLIM2 is highly expressed in CD4+ T cells, which are the preferential targets for HTLV-I infection. Overcoming the strong repression function from the abundant endogenous PDLIM2 expression within CD4+ cells seems to be necessary for ATL progression (Figure 46), since PDLIM2 expression is suppressed to low levels in HTLV-I-transformed T cells and primary ATL samples. Our studies provide an alternative explanation for the long latency and healthy disparity of HTLV-I infection other than accumulation of abundant HTLV-I-infected cells to override the immune response (Figure 46). Furthermore, our studies suggest a potential therapeutic application to prolong or inhibit ATL development by reactivating or re-introducing the PDLIM2.

6.1 NEGATIVE REGULATION OF TAX BY PDLIM2 IN HTLV-I INFECTION AND ATL DEVELOPMENT

HTLV-I encoded oncoprotein Tax, which does not share homology with cellular proto-oncogenes, is crucial for HTLV-I-mediated pathogenesis. Nowadays, the negative regulators of Tax are limited to HTLV-I encoded Rex, p30 and HBZ. Rex suppresses Tax at transcriptional level by inhibiting the generation of mature Tax mRNA (Figure 4); p30 functions to prevent the Tax mRNA nuclear exportation so that inhibiting the Tax translation; HBZ suppress the Tax-

mediated viral transcription by disrupting the formation of Tax-CREB complex at post-translational level (Matsuoka and Jeang, 2007; Nicot *et al.*, 2005). Although serving at distinct regulation levels, they cooperate together and generally facilitate the HTLV-I-induced pathogenesis (Figure 46).

PDLIM2 is a newly identified ubiquitin E3 ligase that could specifically poly-ubiquitinate the nuclear p65 and STAT proteins for proteasomal degradation (Tanaka *et al.*, 2007; Tanaka *et al.*, 2005). Our results demonstrate that PDLIM2 directly interacts with Tax and triggers its proteasomal degradation by promoting the poly-ubiquitination of Tax. In this way, PDLIM2 shuts off Tax-mediated signaling and oncogenesis by suppressing Tax protein levels.

Studies on HTLV-I replication and transmission showed that the HTLV-I viral replication is a transient process and the viral transmission is highly dependent on the clonal expansion of HTLV-I-infected cells (Yoshida, 2010). The transient viral replication involves quick turning on and shutting off of Tax expression. Considering that HTLV-I preferentially infected CD4+ T lymphocytes, which have high levels of endogenous PDLIM2, PDLIM2 induced Tax suppression may contribute to this transient process, therefore supporting the notion that PDLIM2 inhibits Tax-mediated HTLV-I-LTR transcription (Figure 46).

ATL development is always associated with a long latency period typically spanning 40-60 years. The current model stipulates that ATL is generated as HTLV-I-infected cells accumulated from clonal expansion and overcome the threshold of the host immune response. Our studies propose an alternative mechanism involving PDLIM2. We showed that both mRNA and protein levels of PDLIM2 are down-regulated in HTLV-I-transformed T cells as well as the primary ATL cells (Table 2, Figure 30, Figure 31). Interestingly, the purified HTLV-I-infected

cells from HTLV-I carriers and PBMCs from the healthy individuals showed comparable high PDLIM2 expression levels (Figure 31). This indicated that PDLIM2 suppression was associated with HTLV-I-mediated pathogenesis. In other words, only when the HTLV-I-infected cells overrode the suppression function of PDLIM2 such as repressing PDLIM2 expression to a certain level, could those cells exhibit sustained proliferations and result in ATL. Meanwhile, these findings might also distinguish different viral loads between the patients and the carriers: In ATL patients, both Tax and PDLIM2 are repressed, therefore, ATL cells escape from CTL recognition and result in an increased viral load via cell proliferation; however, in carriers, although infected cells initially increase the viral load through clonal expansion, PDLIM2 induced Tax degradation results in a persistent Tax antigen production, therefore allowing CTLs to destroy most of the infected cells in the host, save low Tax expressing cells. This, however, requires further verification by measuring the turnover rate of Tax in infected cells from asymptomatic HTLV-I carriers. If the above-mentioned hypothesis is correct, PDLIM2 serves as the first primary intrinsic defender for HTLV-I infection, supported by the findings that the re-introduction and reactivation of PDLIM2 lead to inhibited oncogenicity and cell death of HTLV-I-transformed T cells, respectively (Table 1, Figure 33, Figure 34). The evidence suggests that PDLIM2 plays a role in innate immune system to prevent the viral infection and cancer development, especially in the case of HTLV-I (Figure 46).

Based on the key role of Tax in HTLV-I-mediated leukemogenesis, we proposed that the expression of PDLIM2 and Tax are tightly counterbalanced in infected cells and that such counterbalance may determine the fate of HTLV-I infection. To this regard, we thought interesting to determine whether Tax could act as a transcriptional repressor of PDLIM2. Unexpectedly, the suppression of PDLIM2 was largely Tax-independent, based on the facts that

both short-term and long-term induction of Tax caused minor changes in PDLIM2 expression (Figure 35). Consistent with that, the cells from asymptomatic HTLV-I carriers expressed both PDLIM2 and Tax, while the ATL cells without Tax expression still presented suppressed expression of PDLIM2 (Figure 31). Although repression of PDLIM2 was largely Tax-independent, the initial infection and replication may be determined by the outcome of PDLIM2-mediated Tax repression so to affect the subsequent cell transformation (Figure 46).

Additionally, Tax expression is also modulated by the immune response, genetic alteration, DNA hyper-methylation, and 5'LTR deletion, in a negative selected manner. The CTL response is the primary immune response to HTLV-I infection by targeting HTLV-I Tax. Correlated with that, the transient expression of Tax helps HTLV-I infection from escaping the immune response. At the meanwhile, the HTLV-I-infected cells with suppressed Tax are negatively selected and survive. PDLIM2 and CTLs may cooperate together to defend the HTLV-I infection. PDLIM2 may serve as a primary defender to suppress viral replication and HTLV-I-mediated cell transformation by inhibition of Tax; while for the cells overcome the suppressive function of PDLIM2, CTLs recognize and eliminate the infected cells. In other words, suppression of both PDLIM2 and Tax expression might be required for ATL development, consistent with the observations that both PDLIM2 and Tax are suppressed in ATL samples (Figure 46).

Recent studies on HTLV-I HBZ gene suggest that HBZ inhibits p65-mediated NF-κB signaling through the activation of PDLIM2 expression; an observation which is in line with the PDLIM2 contribution to HTLV-I-mediated pathogenesis (Zhao *et al.*, 2009). However, there are important flaws within that study that need to be pointed out here. First, all their evidence is

based on the over-expression of proteins. Second, even if HBZ can suppress p65-mediated NF-κB activation, an important paradox remains as nearly all primary ATL cells have HBZ expression and exhibit constitutive activation of NF-κB. Third, our results do not support the notion that HBZ-mediated NF-κB suppression involves activation of PDLIM2. Indeed, PDLIM2 is shown to be largely repressed in HTLV-I-transformed T cells and ATL cells; however, those cells still present HBZ expression. Fourth, PDLIM2 expression does not exhibit differences between HTLV-I carriers and the healthy controls, while cells from HTLV-I carriers present HBZ expression. Therefore, the associations between HTLV-I HBZ, PDLIM2 and NF-κB need to be further investigated. However, this study does offer an explanation for the repression of Tax in ATL cells, by which HBZ activates the expression of PDLIM2 and PDLIM2 subsequently functions to degrade Tax.

6.2 MOLECULAR DETERMINANTS WITHIN PDLIM2 TO REGULATE HTLV-I TAX AND PDLIM2 ITSELF

Table 3. Summary of PDLIM2 mutants' functions on Tax regulation.

PDLIM2 Mutants	Tax				
	Interaction	**Accelerated Turnover**	**Promoted Poly-ubiquitination**	**Subcellular Distribution**	**Suppressed Tumorigenesis**
WT	+	+	+	+	+
Δ PDZ	+	+	+	+	+
Δ LIM	+	-	-	-	-
Δ PDZ/LIM	+	-	-	-	ND
Δ 79-278	-	-	-	-	ND
Δ 195-207	+	+	+	+	ND
Δ 243-253	-	-	-	-	-
Δ 258-278	+	+	ND	+	+
Δ 236-240	+ (weak)	ND	ND	ND	ND
Δ 241-245	-	ND	ND	ND	ND
Δ 246-250	-	ND	ND	ND	ND
Δ 251-255	-	ND	ND	ND	ND
LL241/242PP	-	-	-	-	-
EE249/250PP	-	-	-	-	-

We defined a putative α-helix, located within the internal disordered amino acids 236-254 of PDLIM2, as the Tax-binding motif. PDLIM2-mediated suppression of Tax largely depends on both the Tax-binding motif and its C-terminal LIM domain (Table 3). As indicated in the Chapter 3, multiple sequences within Tax might be involved in PDLIM2 interaction (Figure 23). In contrast, both the LIM domain and the TBM are required to trigger the poly-ubiquitination, proteasomal degradation and subcellular redistribution of Tax by PDLIM2 (Table 3). We showed that selective disruption of the TBM leads to PDLIM2 defects in tumor suppression (Table 3). As a matter of fact, the LIM domain was essential for PDLIM2-mediated Tax repression, while the N-terminal PDZ domain of PDLIM2 was dispensable. These studies dissect functional sequences within PDLIM2 and their roles in regulating HTLV-I oncoprotein Tax.

In the following section I will discuss more on PDLIM2, beyond its effects on Tax. PDLIM2 is ubiquitously expressed in cytoplasm, nucleoplasm and insoluble nuclear fractions and has the ability to interact with cytoskeletal proteins like α-actinin and Filamin A (Torrado *et al.*, 2004). It remains to be shown how PDLIM2 comes to be distributed within distinct subcellular compartments and the relationship between its different subcellular location and biological functions. Our studies suggest that the distinct domains or motifs contribute to the cellular localization of PDLIM2. The deletion mutant of PDZ domain showed an increase in the cytoplasm and a decrease in the nucleoplasm but with no obvious effect in the insoluble nuclear expression, compared to the WT PDLIM2 (Figure 27). Interestingly, although the LIM domain of PDLIM2 is not required for binding to the cytoskeleton, it is involved in targeting PDLIM2 to the insoluble nuclear insoluble fraction. The deletion of the LIM domain leads to dramatic decreased expression of PDLIM2 in the insoluble nuclear fraction and elevated expressions in the cytoplasm and nucleoplasm (Figure 27). Those findings partially explain why the LIM

domain is required for PDLIM2 to alter Tax subcellular distribution as well as trigger Tax proteasomal degradation, while it is dispensable for PDLIM2-Tax interaction. Such evidence also challenge the previous reports suggesting that PDLIM2 triggers p65 degradation in the PML bodies, since their conclusion was based on the inability of the PDLIM2 mutant ΔLIM to co-localize with p65 in PML bodies, which fractionate in the insoluble faction (Tanaka *et al.*, 2007).

Consistent with these results, deletions of both PDZ and LIM domains lead to exclusive expression of PDLIM2 in the cytoplasm, whereas deletion of the entire region between the PDZ and LIM domains results in a dramatic increase in the insoluble nuclear fraction, with a significant decrease in nucleoplasmic PDLIM2 protein (Figure 27). On the other hand, disruption of the TBM or small internal deletions of the middle region of PDLIM2 had no effect on its subcellular distribution (Figure 27). The evidence suggests that distinct domains within PDLIM2 functioned differently in determining PDLIM2 subcellular expression and regulation of Tax.

Importantly, it should be realized that all the former and above findings are based on the exogenous over-expressed PDLIM2. Under physiological conditions, the endogenous PDLIM2 is dominantly found in the nucleus other than in the cytosol (Healy and O'Connor, 2009), in agreement with the fact that PDLIM2 promoted the degradations of nuclear p65 and Tax (Tanaka *et al.*, 2005). Moreover, the sequestration of PDLIM2 to the cytosol results in increased nuclear NF-κB activity, further support the notion that PDLIM2 suppressed p65-mediated NF-κB in the nucleus (Healy and O'Connor, 2009). Nevertheless, regardless of whether the subcellular localization of PDLIM2 is determined by sequences within PDLIM2 itself or by PDLIM2 partners with transporting abilities, the determinants for PDLIM2 subcellular distributions should be further characterized in the future studies.

6.3 DETERMINANT FACTORS IN TAX-MEDIATED TUMORIGENESIS

We sought to reconcile how Tax binding deficient mutant forms of PDLIM2 failed to suppress Tax-mediated anchorage-independent colony formation *in vitro* and tumor growth in SCID mice, if PDLIM2 mutant in Tax-binding retained the ability to suppress p65-mediated NF-κB activation. Is p65-mediated NF-κB activation a key player in Tax-mediated oncogenesis?

Studies on Tax highlighted the importance of NF-κB in Tax-mediated tumorigenesis: Tax induced NF-κB is essential for some skin diseases, and the transduction of super-suppressor form of IκB reverses the malignancy of ATL cells *in vitro*. Considering that suppression of Tax-mediated NF-κB by PDLIM2 involves both p65-dependent and independent mechanism, it is not surprising that both mechanisms contribute to Tax-mediated tumorigenesis. This is supported by the interrelation between the tumor suppression function of PDLIM2 and its ability to interact with Tax (Table 3).

Additionally, our studies had shown that PDLIM2 directly functions to regulate the Tax protein level. This also explains the distinct effect of PDLIM2 mutants in suppression of Tax-mediated tumorigenesis. The function-deficient PDLIM2 mutants (ΔLIM, Δ243-253, LL241/242PP, EE249/250PP) failed to trigger Tax proteasomal degradation, while WT and the function-competitive PDLIM2 mutants (ΔPDZ, Δ258-278) regulated Tax by suppressing Tax levels, resulting in an insufficient Tax signaling response and inhibited oncogenesis. This is supported by the results from detection of the Tax protein levels in both transformed cells and induced xenografts. The high expression of PDLIM2 reduces Tax expression and leads to a nearly total blockage of Tax-mediated tumor growth.

To investigate the role of Tax downstream signaling, we generated the Rat-1 cells with a relatively high expression of Tax in the presence of PDLIM2 WT by intending to use the PDLIM2 under a weaker promoter. Although Tax protein was still relatively low in the presence of WT PDLIM2, the difference was not that significant (Figure 29B). Furthermore, our results from colony formation assays confirmed the suppressive function of PDLIM2 (Table 3). This evidence demonstrated PDLIM2 reduced Tax protein levels as well as Tax downstream signaling circuits play crucial roles in determine Tax-mediated cellular transformation. In other words, both the Tax protein level and the Tax downstream signaling by PDLIM2 are involved in Tax-mediated oncogenesis. Of note, PDLIM2-mediated subcellular distribution of Tax may also be part of Tax signaling and contributes to the events.

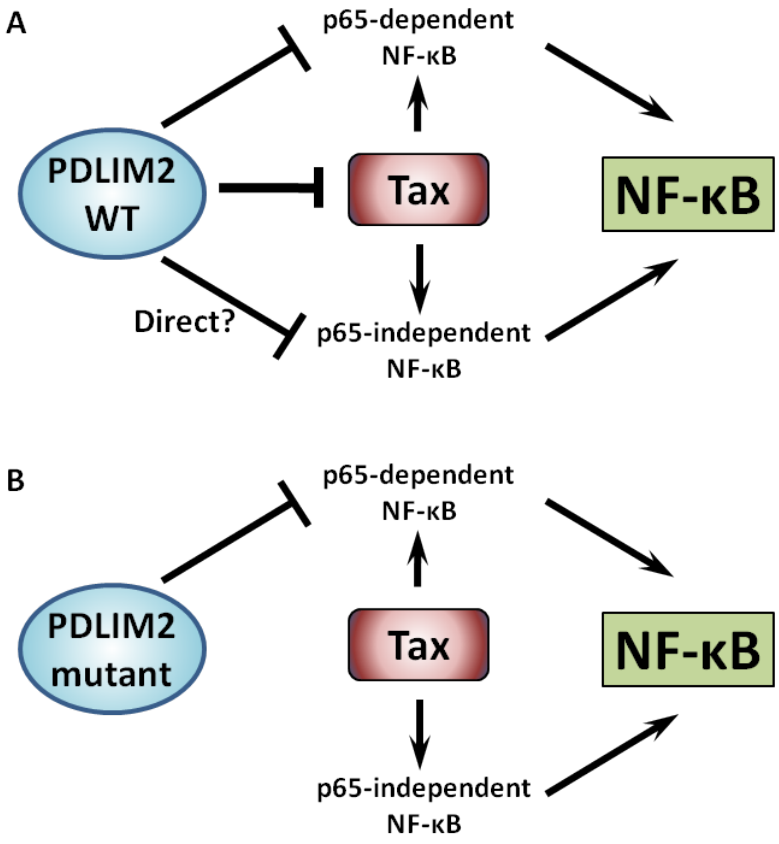

Figure 47. Model of PDLIM2's function in Tax-mediated NF-κB activation.

Those findings therefore suggest that: 1) PDLIM2-mediated inhibition of NF-κB activation involves both p65-dependent and p65-independent mechanisms. And both of them contribute to the suppression of the Tax-mediated NF-κB as well as the oncogenesis by PDLIM2. 2) PDLIM2 degrades Tax, results in the low level of Tax, which is correlated with the suppressed Tax-mediated tumorigenesis by PDLIM2. 3) PDLIM2 affects the subcellular distribution of Tax, which may result in the trapping and inactivation of Tax at certain cellular locations.

Based on that, we proposed a model involves the p65-independent NF-κB activation in Tax-mediated NF-κB signaling by PDLIM2. The WT PDLIM2 suppresses p65-dependent NF-κB activation by targeting both p65 and HTLV-I Tax for proteasomal degradation. Although there is no evidence that whether WT PDLIM2 can directly affect Tax-mediated p65-independent NF-κB activation, the WT PDLIM2 inhibits p52-dependent p65-independent NF-κB activation by suppressing Tax. To the contrary, the Tax-binding-defective mutant of PDLIM2 still retains the ability to degrade p65 and alter p65 subcellular distributions, so that to terminate Tax-mediated p65-dependent NF-κB activation. However, since it fails to trigger poly-ubiquitination and proteasomal degradation of Tax, the Tax-binding defective mutant is unable to attenuate Tax-mediated p65-independent NF-κB signaling as well as Tax-mediated LTR activation. Our studies dissected p65-dependent NF-κB in Tax-mediated NF-κB activation as well as Tax-mediated oncogenesis.

6.4 RE-INTRODUCTION AND REACTIVATION OF PDLIM2 IN CANCER

PDLIM2 plays roles in termination of STAT and NF-κB signaling pathways. The findings here demonstrated that PDLIM2 also targets HTLV-I Tax for the proteasomal

degradation, so as to inhibit Tax-mediated tumorigenesis. Importantly, both mRNA and protein levels of PDLIM2 were down-regulated in HTLV-I-transformed T cells and the primary ATL samples. Although the repressive mechanism remains unclear, it seems that the down-regulation of PDLIM2 is one fundamental mechanism in HTLV-I Tax-mediated cell transformation and ATL development, especially since the re-introduction of PDLIM2 in HTLV-I-transformed T cells is able to reverse tumor growth in animal models.

As discussed in Chapter 4, our studies further indicated that HTLV-I-mediated PDLIM2 repression involved DNA hyper-methylation and a potential link with DNMT1 and DNMT3b. DNMT1 and DNMT3b, but not DNMT3a, were highly expressed in these malignant T cells. Furthermore, PDLIM2 mRNA expression levels increased in the HTLV-I-transformed T cells following cellular exposure to the DNMTs inhibitor. Meanwhile, the elevated expression levels of DNMTs were correlated with the increased PDLIM2 mRNA levels, generally but imperfectly. One plausible explanation is that the epigenetic regulation is a complex process cooperated with other factors. The repression of a particular gene not only requires the availability of individual DNMTs but also depends on many other cellular factors including histone deacetylase 1 (HDAC1) and histone methyltransferase SUV39H1 (Luczak and Jagodzinski, 2006). Importantly, such reactivation of PDLIM2 by 5-aza-dC was associated with the cell death of HTLV-I-transformed T cells, suggesting a novel approach for ATL therapy.

Independently of HTLV-I related diseases, multiple other NF-κB related cancer such as breast, colon and lung cancer cells exhibit low PDLIM2 expression levels (Qu *et al.*, 2010a; Qu *et al.*, 2010b). The re-introductions of PDLIM2 presented potential tumor suppression functions in many tested cancer cells, suggesting PDLIM2 down-regulation as a general mechanism in

cancer progression and as a new cancer biomarker (Loughran *et al.*, 2005; Qu *et al.*, 2010a; Qu *et al.*, 2010b; Yan *et al.*, 2009). Unfortunately, the mechanism by which *PDLIM2* gene is suppressed in cancer cells is unclear. It is reported that 8p21, which is the genomic locus of *PDLIM2* gene, frequently undergoes allelic loss in ovarian and prostate cancers. Another possibility is the epigenetic suppression of PDLIM2, as RIL protein, a family member of PDLIM2 that shows tumor suppression function, is repressed by DNA methylation in many cancers (Boumber *et al.*, 2007; Brown *et al.*, 1999; Swalwell *et al.*, 2002). Our studies on colon cancer and breast cancer provide evidence that the *PDLIM2* promoter was hyper-methylated in those malignant cells and such hyper-methylation at *PDLIM2* promoter directly contributed to the activation of the *PDLIM2* gene (Qu *et al.*, 2010a; Qu *et al.*, 2010b). Importantly, more evidence from our current studies on PDLIM2-deficient mice indicated that those mice generated spontaneous tumors, giving hints that PDLIM2 might also serve as an inhibitor of oncogene (Unpublished data).

6.5 CLOSING REMARDS AND FUTURE DIRECTIONS

In this dissertation, we investigated the regulatory mechanism of HTLV-I Tax by PDLIM2. PDLIM2 serves as the first intrinsic negative regulator of Tax, which is crucial for HTLV-I viral infection, cell transformation and tumorigenesis. Such Tax suppression by PDLIM2 mainly results from PDLIM2 promoted poly-ubiquitination and proteasomal degradation of Tax. Furthermore, results showed that both the LIM domain and the TBM within PDLIM2 were responsible in modulating Tax. Importantly, we showed that HTLV-I and PDLIM2 were counterbalanced. Whereas HTLV-I suppressed PDLIM2 expression at the

transcription level by Tax-independent mechanisms, PDLIM2 promoted Tax turnover at protein levels. Based on those findings, we proposed that the counterbalance between Tax and PDLIM2 would determine the outcome of HTLV-I infection, and contribute to the long latency of ATL progression. Our studies therefore suggested a novel therapeutic strategy for cancer and other diseases associated with HTLV-I infection and/or PDLIM2 deregulation, and provide valuable insights into the leukemogenesis, long latency and cancer health disparities of HTLV-I. Additionally, evidence showed that re-introduction and reactivation of PDLIM2 in HTLV-I-transformed T cells and other cancer cells reverse the malignancy and induce cell death, supporting the potential general tumor suppressive function of PDLIM2.

Regarding the p65 regulation by PDLIM2, further preliminary data showed that PDLIM2-p65 and PDLIM2-Tax served in separated complexes, and PDLIM2 preferentially target Tax other than p65 in the presence of Tax. Although the binding capacity of PDLIM2 towards Tax did not influence PDLIM2-mediated p65-dependent NF-κB activation, the PDLIM2-Tax interaction was associated with the repressive function of PDLIM2 and the outcome of Tax-mediated tumorigenesis. Additionally, we found that p65-independent NF-κB activation by Tax, at lease p52-mediated NF-κB activation, was involved in PDLIM2-suppressed Tax-induced NF-κB activation and Tax-mediated oncogenesis. To summarize, PDLIM2 suppresses Tax-mediated signaling at different levels: for p65-dependent signaling, PDLIM2 directly function at p65 level; for p65-independent mechanisms, PDLIM2 exerts the role in modulating the Tax protein level. Considering that Tax failed to transform p65-deficient MEF *in vitro*, our data indicates that both p65-dependent and p65-independent mechanisms were required for Tax-mediated tumorigenesis.

Unfortunately, the data presented by current studies failed to provide a clear picture regarding PDLIM2, Tax, p65 as well as a conclusive mechanism by which Tax exerts its oncogenic effects. Various questions remain unanswered.

One of the most intriguing questions pertains the infectious feather of HTLV-I in hematopoietic stem cells (HSCs). Recent studies found that HTLV-I could infect human HSCs *in vitro*. After introducing those infected cells into NOD/SCID mice, the mice generated CD4+ T cell lymphomas with the similar phenotype as ATL, indicating a new mechanism for ATL development (Banerjee *et al.*, 2010a; Banerjee *et al.*, 2008; Banerjee *et al.*, 2010b). Importantly, transduction of oncoprotein Tax into HSCs by lenti-viral infection, and subsequently inoculation in NOD/SCID mice showed similar tissue distribution patterns as those HTLV-I-induced lymphomas. Interestingly, compared to almost all other cells involved in immune response including CD4+, CD8+, CD14+, CD19+, CD33+ and CD56+ cells, the HSCs with CD34+ expressing exhibits largely repressed PDLIM2 expression (unpublished data). In other words, HSCs might be the Achilles Heel in defending HTLV-I infection and bypass the high expression of endogenous PDLIM2 in CD4+ T cells. Following proliferation and differentiation, a large population of HTLV-I-infected cells develops and provokes the onset of ATL. This new cancer stem cell model explains well the monoclonal expansion of ATL but further requires supporting evidence.

Further issues remain under investigation. These include: 1) Whether PDLIM2 is the E3 ligase for Tax *in vivo*? Direct evidence would only come from *in vitro* reconstituted ubiquitination assays using recombinant proteins of ubiquitin, E1 and E2 enzymes. Considering the distinct biological functions of K48-linked and K63-linked poly-ubiquitination as well as

their roles in NF-κB regulation, this assay would also distinguish those different modifications on Tax. 2) Are other NF-κB family members and NF-κB complexes involved in the indicated p65-independent NF-κB activation in the presence of Tax? To address this issue, deficient cells of each NF-κB members need to be used. However, as described above, the differences of WT PDLIM2 and Tax binding defective mutant would appear only under p65-knockout condition since p65 is the major target of PDLIM2. Thus, the siRNA or shRNA to knocking down expression of p65 or either of the NF-κB members would be utilized. 3) Which key factor(s) determine Tax-mediated cell transformation and tumor growth? This is a difficult question given the complex biological functions of Tax. However, I believe that the difference in protein profiles in PDLIM2 expressing and non-expressing cells would provide useful information. 4) Other than the functions on Tax and NF-κB, PDLIM2 is also associated with cell migration and cell adhesion. What is the effect of those additional functions of PDLIM2 on tumor repression? Cell migration and cell adhesion are always associated with the active state of cell and metastasis. Invasion assays using transwells and detection of invasion related gene would give hints regarding this issue. 5) Is demethylation a key factor to reactivate PDLIM2? What are the side effects of PDLIM2 re-introduction and reactivation? Are those effects the real responsible factors for the tumor suppression function of PDLIM2? Clues would be easily got via gene or protein arrays. Taken advantage of the PDLIM2 dominant negative form, PDLIM2 ΔLIM, would distinguish the potential role of discovered factors. 6) Is PDLIM2 genuinely involved in ATL progression and how PDLIM2 regulates p65 in HTLV-I present condition? As described before, PDLIM2 is largely repressed in HTLV-I-transformed T cells and ATL cells, while the normal PBMCs and HTLV-I carriers still exhibit high levels of endogenous PDLIM2. It is plausible to assume that suppression of PDLIM2 occurs during the ATL development. One useful model is

that the radio-inactive MT-2 could transform normal PBMCs *in vitro* and mimic the ATL development without CTL stress. Thus, detection of PDLIM2 at different time points during the transforming progress would provide valuable clues.

PDLIM2 played pivotal roles in cellular signaling, immune, inflammatory response, and oncogenesis. I believed that it would be a new target for cancer treatment and a hot-spot in cancer research. Studies on PDLIM2 will provide insights into leukemogenesis, long latency and cancer health disparities of HTLV-I as well as the general tumor suppressive role of PDLIM2.

APPENDIX A.

PUBLICATIONS

1. Fu J, **Yan P**, Li S, Qu Z and Xiao G. (2010) Molecular determinants of PDLIM2 in suppressing HTLV-I Tax-mediated tumorigenesis. ***Oncogene*.** (Accepted and in press)

2. Qu Z, Fu J, **Yan P**, Hu J, Cheng SY, Xiao G. (2010) Epigenetic repression of PDZ-LIM domain-containing protein 2: implications for the biology and treatment of breast cancer. ***J Biol Chem*.** 285(16): 11786-92.

3. **Yan P*,** Qu Z, Fu J, Jiang J, Grusby MJ, Smithgall TE, and Xiao G. (2010) DNA methylation-dependent repression of PDZ-LIM domain-containing protein 2 in colon cancer and its role as a potential therapeutic target. ***Cancer Research*.** 70(5): 1766-72. (*Contribute equally)

4. **Yan P**, Qu Z, Ishikawa C, Mori N, and Xiao G. (2009) Human T-cell leukemia virus type I–mediated repression of PDZ-LIM domain-containing protein 2 involves DNA methylation but independent of viral oncoprotein tax. ***Neoplasia.*** 11(10): 1036-41

5. **Yan P**, Fu J, Qu Z, Li S, Tanaka T, Grusby MJ, and Xiao G. (2009) PDLIM2 suppresses human T-cell leukemia virus type I Tax – mediated tumorigenesis by targeting Tax into the nuclear matrix for proteasomal degradation. ***Blood.*** 113(18): 4370-80

6. **Yan P**, Qing G, Qu Z, Wu CC, Rabson A, and Xiao G. (2007) Targeting autophagic regulation of NF-κB in HTLV-I-transformed cells by geldanamycin: implications for

therapeutic interventions. ***Autophagy.*** 3(6): 600-3

7. Qing G, **Yan P**, Qu Z, Liu H, Li H, and Xiao G. (2007) Hsp90 regulates processing of NF-κB2 p100 involving protection of NIK from autophagy-mediated degradation. ***Cell Res.*** 17(6): 520-530. **Cover Article.**

8. Zhang D, Shao C, Shao M, **Yan P**, Wang Y, Liu Y, Liu W, Lin T, Xie Y, Zhao Y, Lu D, Li Y, and Jin L. (2007) Effect of Mu-Opioid Receptor Gene Polymorphisms on Heroin-Induced Subjective Responses in a Chinese Population. ***Biol Psychiatry.*** 61(11):1244-1251.

9. Qing G, **Yan P**, Qu Z, and Xiao G. (2006) Hsp90 inhibition results in autophagy-mediated proteasome-independent degradation of IκB kinase. ***Cell Res.*** 16(11): 895-901. **Also See Comment in *Cell Res.*** 16(11): 855-856.

10. Li Y, Shao C, Zhang D, Zhao M, Lin L, **Yan P**, Xie Y, Jiang K, and Jin L. (2006) The Effect of Dopamine D2, D5 Receptor and Transporter (SLC6A3) polymorphisms on the Cue-Elicited Heroin Craving in Chinese. ***Am J Med Genet B Neuropsychiatr Genet.*** 141(3):269-273.

11. Li Y, Li Q, Zhang D, Jin W, Xu C, Sheng H, Liu Y, Lu G, Yu J, **Yan P**, Xie Y, Lu D, Nebert D, Harrison D, Huang W, and Jin L. (2006) Mitochondrial aldehyde dehydrogenase-2 (ALDH2) Glu504Lys polymorphism contributes to the variation in efficacy of sublingual nitroglycerin. ***J Clin Invest.*** 116(2):506-511.

12. Li Q, Li Y, Shao M, Li G, Zhang D, **Yan P**, Ding D, Xie Y, Jin L, and Lu D. (2004) Cloning and Expression of Different Genotypes of Human Aldehyde dehydrogenase. ***J Fudan Univ.*** 43(6):1079-1083.

APPENDIX B.

PRIMERS FOR GENERATING PDLIM2 MUTANTS

mPDLIM2 Δ79-278

Forward: 5’-CTCACCCCTAAGACTGAAGCTTCACACCTGTG

Reverse: 5’-CACAGGTGTGAAGCTTCAGTCTTAGGGGTGAG

mPDLIM2 Δ111-145

Forward: 5’-GCTCCCTGAGGACAGCGCTGTCTAAAGAG

Reverse: 5’-CTCTTTAGACAGCGCTGTCCTCAGGGAGC

mPDLIM2 Δ195-207

Forward: 5’-GCGACTCCGCGGTGAGGTTCAGCAGTTTGGATC

Reverse: 5’-GATCCAAACTGCTGAACCTCACCGCGGAGTCGC

mPDLIM2 Δ242-253

Forward: 5’-GCTCTTTTCGACTGTTAACACCTGCCTTTGTG

Reverse: 5’-CACAAAGGCAGGTGTTAACAGTCGAAAAGAGC

mPDLIM2 Δ258-278

Forward: 5’-GGCACACCTGCCTTTAAGCTTCACACCTGTG

Reverse: 5’-CACAGGTGTGAAGCTTAAAGGCAGGTGTGCC

mPDLIM2 Δ216-228

Forward: GCAGTTTGGATCT*C*GAGGAAGGACGGGCCGCCCCAA

Reverse: TTGGGGCGGCCCGTCCTTCCTC*G*AGATCCAAACTGC

mPDLIM2 Δ238-250

Forward: CCGCCCCAAGGCAGTCC*TCG*AGAGGTGGCACACCTG

Reverse: CAGGTGTGCCACCTCT*C*GAGGACTGCCTTGGGGCGG

mPDLIM2 L78R

Forward: GCGCCTCACCCCTACGTCGACAGCTGGACCGGTC

Reverse: GACCGGTCCAGCTGTCGACGTAGGGGTGAGGCGC

mPDLIM2 L80K

Forward: CCCTAAGACTGCAGAAGGACCGGTCCCAAA

Reverse: TTTGGGACCGGTCCTTCTGCAGTCTTAGGG

mPDLIM2 LL78/80RK

Forward: CTCACCCCTAAGACGACAGAAGGACCGGTCCCAAA

Reverse: TTTGGGACCGGTCCTTCTGTCGTCTTAGGGGTGAG

mPDLIM2 CC310/313SS

Forward: CTGGCTGCTACACTAGCGCTGACAGTGGGCTGAACCTG

Reverse: CAGGTTCAGCCCACTGTCAGCGCTAGTGTAGCAGCCAG

mPDLIM2 △231-235

Forward: AGAACCGCCAGGGAAGATCTAGCTCTTTTCGACT

Reverse: AGTCGAAAAGAGCTAGATCTTCCCTGGCGGTTCT

mPDLIM2 △236-240

Forward: CCGCCCCAAGGCAGCTGTTACAGGAAGCCTT

Reverse: AAGGCTTCCTGTAACAGCTGCCTTGGGGCGG

mPDLIM2 △241-245

Forward: TCCAGCTCTTTTCGTCTAGAGGCTGAGGAGAG

Reverse: CTCTCCTCAGCCTCTAGACGAAAAGAGCTGGA

mPDLIM2 △246-250

Forward: TCTTACAGGAAGCCCGGGGTGGCACACCTGC

Reverse: GCAGGTGTGCCACCCCGGGCTTCCTGTAAGA

mPDLIM2 △251-255

Forward: TTGGAGGCTGAGGAAGCTTTTGTGCCCAGCTC

Reverse: GAGCTGGGCACAAAAGCTTCCTCAGCCTCCAA

mPDLIM2 △256-260

Forward: AGAGGTGGCACACCTTCGCTGAGCTCCCAG

Reverse: CTGGGAGCTCAGCGAAGGTGTGCCACCTCT

mPDLIM2 △261-265

Forward: GCCTTTGTGCCCAGCGCTTCCTTGCCCACC

Reverse: GGTGGGCAAGGAAGCGCTGGGCACAAAGGC

mPDLIM2 △265-271

Forward: GCTCGCTGAGCTCCCGGGCCTTGGCCACT

Reverse: AGTGGCCAAGGCCCGGGAGCTCAGCGAGC

mPDLIM2 △272-278

Forward: CCTTGCCCACCTCCAAGCTTCACACCTGTGAGAA

Reverse: TTCTCACAGGTGTGAAGCTTGGAGGTGGGCAAGG

mPDLIM2 LL241/242PP

Forward: TCCAGCTCTTTTCGGCCGCCGCAGGAAGCCTTGGA

Reverse: TCCAAGGCTTCCTGCGGCGGCCGAAAAGAGCTGGA

mPDLIM2 EE249/250PP

Forward: AAGCCTTGGAGGCTCCGCCGCGGGGTGGCACACCTGC

Reverse: GCAGGTGTGCCACCCCGCGGCGGAGCCTCCAAGGCTT

*Some mutants were not mentioned in the dissertation, but the constructs were generated and tested in the studies.

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